The Tumor Suprressor APC: Nuclear Functions and Regulation by Heat Shock Response

By

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Abstract

Because mutation of the tumor suppressor *APC* initiates ~80% of all colorectal cancers, understanding APC function is central for better diagnostic, preventive, and therapeutic strategies for the disease. *In-vitro* studies have indicated that APC shuttles between the cytoplasm and nucleus, using two nuclear localization signals (NLS) and five nuclear export signals (NES). To better understand the role of nuclear APC, our lab made a mouse model with mutations in both NLS (Apc^{mNLS}) sequences. In this dissertation, I report higher Wnt signalling and increased proliferation in intestinal epithelial cells from $Apc^{mNLS/mNLS}$ mice, and observe that these mice are more susceptible to colitis-induced colon tumorigenesis. Furthermore, Apc^{Min} mice, a well-characterized Apc mouse model that carries an *Apc* truncation mutation, have increased intestinal polyp multiplicity, size, and proliferation index when they also carry the Apc^{mNLS} allele. Taken together, these data support a role for nuclear Apc in cell proliferation, inhibition of Wnt signalling, and tumor suppression. $Apc^{mNLS/Min}$ mice also display extra-intestinal phenotypes, including enhanced mammary tumorigenicity and more severe anaemia, than in $Apc^{Min/+}$ mice, suggesting a role for nuclear Apc in other tissues.

My studies also identified and characterized a polymorphism in the promoter of the *Pla2ga2* (*Mom-1*) gene that might be responsible for the attenuated phenotype observed in $Apc^{Min/+}$ mice in some genetic backgrounds. I developed a simple, reliable, PCR-based test for this polymorphic allele that will allow easy screening of mouse colonies.

The mechanisms by which cellular APC levels are regulated are not completely understood. In this dissertation, I show that induction of the heat-shock response

increases APC levels both in colon cancer cell lines and in mouse intestinal epithelial cells. I tested two compounds to induce the heat-shock response and found altered tumor multiplicity, size, and regional distribution in two mouse models with different germline mutations in *Apc*. I also showed that a novel non-toxic heat-shock response inducer, KU-32, protects against colitis-mediated tumorigencity in mice. I propose that regulation of APC levels via heat-shock response contributes to many aspects of APC and intestinal tumor biology, and can serve as a novel molecular target for prevention and treatment of colorectal cancer.

This work is dedicated to my parents Helmy Zeineldin and Zainab Barghash

إلى أبي وأمي الحبيبين

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List of Abbreviations

17-AAG	17-N-Allylamino-17-demethoxygeldanamycin
a.a.	Amino acid
AFAP	Attenuated Familial Adenomatous Polyposis
AOM	Azoxymethane
APC	Human *Adenomatous Polyposis Coli* gene
APC	Human Adenomatous Polyposis Coli protein
Apc	Mouse *Adenomatous polyposis coli* gene
Apc	Mouse Adenomatous polyposis coli protein
ARMS	Amplification refractory mutation system
CAT	Chloramphenicol acetyl transferase
CBC	Complete blood count
ChIP	Chromatin immuno-precipitation
CHRPE	Congenital hypertrophy of retinal pigment epithelium
CIN	Chromosomal instability
Cox-1	Cyclooxygenase-1
Cox-2	Cyclooxygenase-2
CRC	Colorectal cancer
CtBP	C-terminal binding protein
DAPI	4',6-diamidino-2-phenylindole
DSS	Dextran sodium sulfate
EMSA	Electrophoresis mobility shift assay
ENU	N-ethyl-N-nitrosourea

ES cells	Embryonic stem cells
FAP	Familial Adenomatous Polyposis
FFPE	Formalin-fixed-paraffin- embedded
FISH	Fluorescence in-situ hyberidyzation
GI	Gastrointestinal
HCT	Hematocrit
Hg	Hemoglobin
HIF-1	Hypoxia-induced factor-1
HNPCC	Hereditary non-polyposis colon cancer
HSE	Heat-shock element
HSF1	Heat-shock factor 1
HSP70	Heat-shock protein 70
HSP90	Heat-shock protein 90
IBD	Inflammatory Bowel disease
IL	Interleukin
KAD	Kyoto Apc Deleted rat
LOH	Loss of heterozygozity
LP-BER	Long patch-base excision repair
MCR	Mutation cluster region
MEF	Mouse embryonic fibroblasts
Min	Multiple intestinal neoplasia
MIP-2	Macrophage inflammatory protein 2
mNLS	Mouse nuclear localization signal

Mom	*Modifier of min*
NES	Nuclear export signal
NF-κB	Nuclear factor κB
NLS	Nuclear localization signal
Nos-2	Nitric oxide synthetase-2
NSAID	Non-steroidal anti-inflammatory drugs
OPN	Osteopontin
PBS	Phosphate-buffered saline
PG	Prostaglandins
PIRC	Polyposis in rat colon
Pla2g2a	*Phospholipase A2 group 2a*
RBC	Red blood cells
SAMP	Serine-alanine-methionine-proline
TA cells	Transient-amplifying cells
UTR	Untranslated region
VEGF	Vascular endothelial growth factort

Chapter1

Introduction

Colorectal cancer

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the United States [1]. In 2011, it was estimated that there were ~ 150,000 new colon cancer cases and 50,000 related deaths [1]. Although there have been improvements in CRC diagnosis and management in recent years, the treatment options of CRC, including surgical removal and chemotherapy, are associated with a relatively high morbidity [2]. There are many risk factors that have been associated with CRC, including age, diet, ethnic background, known genetic alterations, family history of the disease, and chronic inflammation in the colon (chronic colitis) [3, 4]. Increased understanding of colon cancer will reveal better preventive, diagnostic, prognostic, and therapeutic strategies.

Genetic susceptibility to CRC

Cancer is a genetic disease that results from accumulation of genetic mutations and epigenetic modifications in the genome [5]. Cancer-related genes are broadly classified as proto-oncogenes and tumor suppressor genes. Proto-oncogenes are genes encoding proteins that promote cellular growth and proliferation. Cancer-associated mutations in proto-oncogenes are usually gain-of-function and drive uncontrolled cellular proliferation. On the other hand, cancer-driving mutations in tumor suppressor genes are usually loss-of-function. Tumor suppressor genes are further divided into “gatekeepers” and “caretakers”. Gatekeeper genes work as breaks on cellular proliferation while caretaker genes are responsible for DNA repair to prevent further accumulation of oncogenic mutations [5]. Mutations in proto-oncogenes and tumor suppressor genes are

usually acquired (somatic) in sporadic cases of cancer. However, mutations can also be inherited (germline) to result in tumor susceptibility phenotypes. The penetrance of these cancer susceptibility phenotypes varies from almost 100% to only slightly increasing the risk for a particular cancer. This genetic-associated risk increase for cancer phenotypes is one basis for familial cancer predisposition [6].

Family history of CRC increases the risk in sporadic cases. In addition there are also relatively well-characterized CRC susceptibility syndromes [7]. Characterizing the genes mutated in these cancer susceptibility phenotypes allows uncovering of various molecular pathways in colorectal carcinogenesis, which permits identification of various preventive, diagnostic, prognostic, and therapeutic targets.

There are many CRC susceptibility syndromes that vary in their mode of inheritance, penetrance, colonic lesion pathology, and extra-colonic phenotypes [8] (Table 1.1). All of these syndromes are autosomal dominant except for MYH-associated attenuated polyposis syndrome, which is autosomal recessive. All of these CRC syndromes are also associated with increased risk for other types of cancer. These cancer susceptibility syndromes result from mutations in gatekeepers (*APC* in familial Adenomatous polyposis (FAP), *BMPR1A* and *SMAD4* in Juvenile polyposis, and *STK11* in Peutz-Jeghers syndrome) and caretakers (*MLH1, MSH2, PMS2,* and *MSH6* in hereditary non-polyposis colon cancer (HNPCC) syndrome, and *MYH* in MYH-associated attenuated FAP) [9-14]. In addition to these characterized CRC susceptibility syndromes, mutations in other genes were also identified in families with increased risk for CRC (see [15] for the list of these genes).

Table 1.1: CRC susceptibility syndromes

Syndrome	OMIM	Gene	Inheritance	Penetrance	Colonic lesions	Extra-colonic manifestations	ref
Familial adenomatous polyposis (FAP)	175100	*APC*	Autosomal Dominant	100% by age 40y	Adenoma	Hepatocellular, duodenal, pancreatic, thyroid and brain carcinoma Desmoids tumors, CHRPE, osteoma	[9; 10]
Peutz-Jegher	175200	*STK11*	Autosomal Dominant	40% by age 70y	Adenoma Hamartoma	Cervical, uterine, breast, testicular and pancreatic carcinoma Skin and mucosal pigmentation, respiratory polyps, hyperestrogenism	[11]
Juvenile polyposis	174900	*SMAD4* *BMPRA1*	Autosomal Dominant	30-60% by age 60y	Adenoma Hamartoma	Gastric, small intestinal and pancreatic adenocarcinoma Hereditary hemorrhagic telangectasia	[12]
Hereditary non-polyposis colon cancer (HNPCC) Lynch syndrome	120435	*MLH1*, *MSH2*, *PMS2* and *MSH6*	Autosomal Dominant	90% by age 74y	Adenoma (small and few)	Gastric, uterine, cervical, brain, renal, pancreatic, biliary cancer Keratoacnthoma and sebaceous adenoma	[13]
MYH-associated attenuated polyposis	608456	*MYH*	Autosomal recessive	80% by age 70y	Adenoma	Duodenal carcinoma CHRPE, desmoid tumors, osteoma, dental anomalies, upper GIT polyps	[14]

CHRPE: congenital hypertrophy of retinal pigment epithelium.

Familial Adenomatous Polyposis (FAP)

Familial adenomatous polyposis (FAP) was the first CRC syndrome to be characterized [8]. The incidence of FAP is different in different population, and is around 1/10,000 in Europe [8]. FAP syndrome is characterized by the growth of tens to thousands of adenomas (polyps) in the colon, and is transmitted as an autosomal dominant trait with almost 100% penetrance by the age of 40. Although these tumors are benign, there is a ~1-5% risk for each tumor to undergo a malignant transformation. Considering the huge number of polyps that typically develop in FAP patients, without intervention, CRC is almost inevitable. FAP patients are usually asymptomatic until the age of late twenties, when the polyps are large enough to manifest [16]. In addition to CRC, FAP patients have higher risks of developing other cancers such as hepatic, pancreatic, thyroid, and duodenal carcinomas. In addition, FAP patients may also present with a medulloblastoma brain tumor; Turcot syndrome describes the co-occurrence of CRC and medulloblastoma [17, 18]. FAP patients also develop other extra-colonic manifestations including desmoid tumors, dental anomalies, and congenital hypertrophy of retinal pigment epithelium (CHRPE); Gardner syndrome describes the presence of these extra-colonic manifestations with colonic polyps [19]. The only treatment of FAP is surgical removal of the colon, usually in a person's late twenties, to prevent the development of cancer. This prophylactic measure is associated with morbidity and does not affect the morbidity and mortality from other extra-colonic manifestations associated with the condition [16]. Although FAP represents about 1% of all cases of CRC, mapping and characterizing its genetic basis resulted in identifying the main colonic gatekeeper

gene that is mutated not only in this syndrome but in the vast majority of all CRC, the *Adenomatous Polyposis Coli* (*APC*) gene.

Adenomatous Polyposis Coli (APC)

Discovery

APC was identified as the candidate gene for FAP (Gardner syndrome) by two independent groups [20-22]. First, deletion of the 5p21 chromosomal band was detected in a patient with FAP syndrome [23]. Following this cytogenetic finding, genetic linkage confirmed the presence of a FAP candidate gene on 5p21 [24, 25]. The group of Bert Vogelstein at Johns Hopkins University, in collaboration with the Nakamura group at the University of Tokyo, linked several genes present in this chromosomal region (*FER*, *MCC*, *SRP19, TB2* and *APC*) to patients with FAP and colon cancer [21]. All these genes are expressed in the colonic epithelium. Kinzler *et al.* were able to narrow the list to *MCC* and *APC* [21]. Nishisho *et al.* found nonsense mutations in the *APC* gene in patients with FAP and Gardner syndromes [22].

Ray White's group at the University of Utah found a large deletion at the 5p21 locus in 2 unrelated cases with FAP. They found 3 candidate genes in this deletion; *SRP19*, *DP1* (deleted in polyposis 1 which turned out to be *MCC*) and *DP2.5* [20, 26]. The group searched for mutations in these 3 genes in 61 unrelated FAP patients. They characterized exon boundaries of these 3 genes using Northern blots, and screened for mutations in the predicted exons using single-stranded conformational polymorphisms (SSCP) [20]. They found nonsense mutations and small deletions resulting in frameshifts in the *DP2.5* gene, which turned out to be the *APC* gene [20].

Screening sporadic cases of colorectal tumors (benign and malignant) of different sizes showed that mutations in the *APC* gene are present in most of theses cases. In addition, *APC* mutations were also detected in early adenomas and in small tumors. These observations suggest that mutations in *APC* are early, if not the initiating, step in most cases of CRC [27]. In addition, using immunohistochemistry, full-length APC has been detected in normal colon epithelium and cell lines from sporadic tumors from other organs, but not in colonic adenomas, carcinomas, or most CRC cell lines [28]. Considering the severity of the phenotype in FAP patients and the finding that *APC* is mutated early in most cases with sporadic CRC, Kinzler and Vogelstein concluded that APC is the gatekeeper of the colonic epithelium [7]. Fearson and Vogelstein described a genetic model for colorectal carcinogenesis in which different genetic changes in 5 different pathways are associated with morphological progression of cells to invasive carcinoma [29]. The first step in this model is mutation of both *APC* alleles [7, 29].

APC structure and functions

APC gene is formed of 15 exons with the last exon coding for more than three quarters of the protein product [7, 20]. The *APC* gene codes for a large protein, APC, 2843 amino acids in length, and about 312 kD [20]. Later work identified a small exon (exon 10A) that is alternatively spliced in a tissue-non-specific mechanism that adds 18 in-frame codons to the *APC* final mature mRNA[30].

The product of the *APC* gene is a large multi-domain protein that interacts with many proteins and was implicated in many cellular processes (Figure 1.1) [31, 32]. The N-terminal part of APC contains an oligomerization domain, allowing APC to make a homodimer [33]. APC has a region that has sequence similarity with the armadillo

protein (β-catenin homologue in *Drosophila*), and therefore called the armadillo repeat region [21]. The middle region of APC contains four 15-amino acid (aa) repeats and seven 20-aa repeats that are important in binding and destruction of β-catenin (see below) [34-36]. Within the 20-aa repeats are three axin-binding Ser-Ala-Met-Pro (SAMP) repeats [37]. The C-terminal region of APC binds microtubules both directly and via the microtubule-binding domain of EB1 [38, 39]. The most C-terminal region of APC has a PDZ domain that binds to the discs large protein, a component of the tight junction [40].

The most characterized function of APC is to antagonize WNT signaling-induced cellular proliferation by targeting the oncoprotein β-catenin for proteasomal degradation [41]. Two groups demonstrated that APC co-immunoprecipitates with β-catenin [34, 42]. Further work showed that APC forms a cytoplasmic multi-protein complex with AXIN, the serine/ threonine kinase (GSK-3β) and casein kinase 1α (CK1α). This complex phosphorylates and targets β-catenin for proteasomal degradation to maintain a low level of cytoplasmic β-catenin. Canonical WNT signaling inhibits this destruction complex, which results in accumulation of β-catenin (figure 1.2).

WNT signaling is a conserved developmental pathway that plays an important role in cellular proliferation and differentiation [43]. WNT was discovered through cloning of a *Drosophila melanogaster* gene causing a wing defect (*Wingless, Wg*) and also the identification of the integration site of the mouse tumorigenic virus MMTV (*Int1*) [44, 45]. WNT is a group of secreted ligands that bind to serpentine cell-surface receptors of the Frizzled family (FZ). The WNT-FZ complex binds to LRP5/6, Disheveled and AXIN and results in inhibiting the β-catenin destruction complex. Hypophosphorylated β-catenin accumulates in the cytoplasm and translocates to the

nucleus, where it binds to the co-transcriptional factor TCF/LEF to alter the expression of WNT target genes [43]. Most β-catenin-responsive genes are induced e.g. *MYC*, *CyclinD1* and *AXIN2;* other genes are downregulated, however, e.g. *HATH1* [46-49] (for an updated list of WNT target genes see the WNT homepage http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes). The effect of WNT signaling on cells is usually to drive cellular proliferation and maintain an undifferentiated state [43].

WNT signaling plays an important role in maintaining intestinal epithelial architecture [50]. Intestinal epithelium is formed of a single layer of columnar epithelial cells that are arranged in finger-like projection (villi, only in the small intestine) and sac-like invaginations (crypts, in both the small and large intestines); see figure 1.3. It was demonstrated that stromal cells at the crypt base secrete WNT ligands that maintain a gradient concentration of WNT along the length of the crypt. At the crypt base (highest concentration of WNT) intestinal stem cells are located. These stem cells divide to maintain the stem cell population and also produce undifferentiated cells (transient amplifying cells; TA). TA cells further divide until they reach the upper 1/3 of the crypt (with lower WNT concentration) where these cells start to differentiate to various intestinal cell types [43, 45]. It is thought that the inability of APC to antagonize WNT signaling results in continuing proliferation, lack of differentiation and intestinal tumor formation [51].

In addition to antagonizing WNT signaling, APC also has WNT-independent roles in other vital cellular functions, including adhesion, migration, cytoskeleton organization, spindle formation and chromosome segregation [31]. APC co-

immunoprecipitates with the adherens junction proteins α-catenin and E-Cadherin [34]. Full-length, but not truncated, APC co-localizes with microtubules [42] and also concentrates on the leading edge of migrating epithelial cells [52, 53]. At this leading edge, APC forms a complex with microtubules and the RNA binding protein FMRP. FMRP binds to and regulates translation of different RNA transcripts needed for formation of pseudopodial protrusion during cellular migration [52]. In addition, depolymerization of microtubules results in redistribution of APC in the cell, which suggests that APC interacts with microtubules [34, 39]. This interaction involves the C-terminal part of APC and seems to be unrelated to the protein's function to antagonize WNT signaling [54]. APC has also been shown to interact with the microtubule-associated protein EB1 [55, 56]. In addition, the Neufeld lab has shown that APC interacts with the intermediate filaments Lamin B1 and Keratin 81 in cultured cells [57]. Mutations in *APC* have been associated with chromosomal instability in both colon cancer cell lines and mouse embryonic stem cells [58-60]. Moreover, Apc mutations have also been shown to affect the sensitivity for microtubule poisons in cultured cells and mouse intestinal epithelial cells, inhibiting the spindle assembly checkpoint-induced mitotic arrest to low dose of the microtubule poisons [61].

Nuclear APC

In addition to the cytoplasmic functions described above, APC shuttles between the cytoplasm and the nucleus. APC is a large protein that can not diffuse freely into the nucleus [62]. In 1997, Neufeld and White, using immuno-fluorescence and cell fractionation, found that APC is located in both the cytoplasm and the nucleus. Furthermore, they showed that nuclear APC is co-localized with rRNA in the nucleolus

[53]. Zhang *et al.* described 2 monopartite nuclear localization signals in the C-terminal half of APC protein ($NLS1_{APC}$ and $NLS2_{APC}$) [63]. These nuclear localization signals are conserved in human, mouse, rat, and fly APC, and have sequence similarity to the nuclear localization signal seen in the SV-40 large T-antigen. $NLS1_{APC}$ and $NLS2_{APC}$ can target cytoplasmic β-galactosidase to the nucleus [63]. Furthermore, mutation in $NLS1_{APC}$ and $NLS2_{APC}$ results in exclusion of the full-length APC from the nucleus in cultured cells [63]. Galea *et al*. showed that truncated APC that lacks both NLS1 and NLS2 can still accumulate in the nucleus when nuclear export is inhibited by use of leptomycin B1 (LMB1) [64]. They further showed that this NLS1-NLS2-independent nuclear import of APC requires amino acids 302- 625 (in the armadillo repeat region) [64]. On the other hand, nuclear export of APC to the cytoplasm is facilitated by 5 nuclear export signals (NES) [62]. Two of these NES are in the N terminal part, 2 are in the middle region, while the last one lies in the C-terminal region of the APC [62, 65-68].

It has been suggested that nuclear APC has a role in antagonizing WNT signaling in a mechanism that does not involve targeting β-catenin for destruction [62]. Three different mechanisms have been proposed: 1- helping to export β-catenin from the nucleus; 2- sequestering nuclear β-catenin from interaction with the TCF/LEF transcription factor and; 3- facilitating import of transcriptional repressors that inhibit β-catenin transcriptional activity [62]. Evidence supporting model one include the observation that inhibition of nuclear export of APC, via introduction of NES mutations or treatment with the nuclear export inhibitor LMB1, results in accumulation of nuclear β-catenin in cultured cells [66, 68, 69]. In addition, expression of full-length APC with intact nuclear localization signals (NLS1 and NLS2) decreases nuclear β-catenin levels,

and reduces β-catenin transcriptional activity in colon cancer cultured cells that have endogenous truncated APC. This WNT-antagonizing activity of APC appears to be NLS-dependent, since mutation in both NLSs eliminates these effects [69].

The second proposed mechanism by which nuclear APC can dampen β-catenin transcriptional activity is through sequestering nuclear β-catenin from transcription co-factor TCF/LEF [62]. Nuclear APC interacts with nuclear β-catenin [66, 70]. In addition, it has been shown that APC and TCF/LEF bind to the same β-catenin domain. Moreover, a single APC molecule can bind to up to 10 β-catenin molecules [70]. Furthermore, inhibition of nuclear export of APC has been correlated with reduced β-catenin transcriptional activity [65-68]. Considering these data, nuclear APC might compete with TCF/LEF for β-catenin binding, thus decreasing β-catenin transcriptional activity.

In the third model, APC binds to and helps nuclear import of the transcriptional suppressor C terminal-binding protein (CtBP) to antagonize WNT signaling [71]. APC binds to CtBP both *in vitro* and *in vivo*. The nuclear APC/CtBP complex appears to decrease β-catenin transcriptional activity in the nucleus. There is no evidence that CtBP binds to TCF, however, so perhaps CtBP might sequester nuclear β-catenin [71]. Also, chromatin immunoprecipitation (ChIP) results found that the APC/CtBP complex (along with another transcriptional co-repressor) binds to the promoter of c-MYC, a β-catenin transcriptional target [72]. Together these results supporting each of the 3 models suggest that nuclear APC opposes WNT signaling by multiple mechanisms and at levels [62, 72].

Other proposed roles for APC in the nucleus include DNA synthesis, cell cycle regulation, and DNA repair [62]. Our laboratory has shown that APC interacts with Topoisomerase IIα, an enzyme essential in DNA replication and cell cycle progression

[73, 74]. APC interacts with PCNA, FEN-1, and polymerase-β, components in long patch-base excision repair (LP-BER) [75-80], and can affect CREB-C/EBP- mediated transcription [81]. Although the significance is not completely understood, APC appears to directly interact with A/T-rich DNA sequences [82].

Although accumulated evidence demonstrates roles for wild-type APC in the nucleus, two important notes should be considered. First, the above-mentioned data were collected using cultured cells and/or purified proteins, which may not reflect APC's function in living organisms. Second, cancer-associated mutations in APC usually result in deletion of the C-terminal part of the protein, including many important domains in addition to both NLS. This multi-domain loss makes it difficult to dissect the patho-physiologic role of NLS_{APC} in the context of normal homeostasis and carcinogenesis. In view of these limitations, making an animal model with compromised nuclear APC has been necessary to better understand the role of APC in the nucleus.

Mutations in *APC*

Mutations in both *APC* alleles are detected in the majority of colorectal tumors, thus filling Knudson's two-hit hypothesis for tumor suppressor genes [83]. Knudson's two-hit hypothesis explains the apparent paradox that the multiple polyposis phenotype in FAP patients is inherited as an autosomal dominant trait with mutation in only one *APC* allele, while at the cellular level, loss-of-function mutations in both *APC* alleles are required for the development of the adenoma phenotype (recessive) [5]. In FAP syndrome, the patients inherit a mutant copy of *APC,* and polyps are thought to initiate following a somatic mutation in the other wild-type *APC* allele. Considering the large number of crypts in the colon, mutation of the second *APC* allele in an FAP patient

happens in tens to thousands of crypts, resulting in the "multiple polyposis" phenotype [5, 6]. Consistent with this interpretation, in the majority of sporadic colorectal cancers, somatic mutation of both *APC* alleles is required in a single colon epithelial cell in order to initiate colorectal tumorigenesis [84]. This explains why sporadic CRC usually develops as a single lesion and at a later time, relative to tumors in FAP syndrome [5, 84]. There are no reports for germline mutations in both copies of *APC*, which suggests that loss of APC function is lethal early in development. In addition, homozygous mutation of both *Apc* copies is embryonically lethal in mouse, rat, and zebrafish, which reinforces the importance of APC in fetal development [85-87].

Comparing *APC* mutations in tumors from FAP patients suggests that the sites of somatic mutations within the *APC* gene are not random and instead depend on the position of the germline mutations [88]. Most germline *APC* mutations in FAP patients are nonsense mutations or small insertions/ deletions that result in frameshifts and premature stop codons [89, 90]. The same types of mutations are seen as the second *APC* gene mutation in tumors from these patients [89, 90]. Loss of heterozygosity (LOH) refers to loss of the wild-type allele of a gene (usually a tumor suppressor gene in tumor cells) with or without duplication of the mutant allele. LOH results from: 1- somatic non-disjunction and loss of the whole chromosome with or without duplication of the homologous chromosome, 2- deletion of a chromosomal locus or 3- mitotic recombination [6]. The latter mechanism is most commonly seen in FAP patients [91, 92]. It is thought that the second *APC* allele mutation seen in the tumors from FAP patients confers selective advantage for tumor development. As LOH in mammalian genomes seems to be more frequent than the other mutational events including point

mutation, small deletions and insertions [91, 93], the frequency of *APC* LOH seen in tumors from FAP patients with a specific germline *APC* mutation implies the selective advantage of this mutation on tumorigenicity. It has been shown that if the *APC* germline mutation occurs between the first and second 20 a.a. repeats, the somatic mutation usually happens via LOH. Furthermore, if the germline mutation occurs before the first 20 a.a. repeat, the second hit is usually a nonsense mutation between the first and second 20 a.a. repeats with reduced frequency of LOH in these patients. These observations indicate that a truncation mutation in *APC* after the first 20 a.a. repeat is not only enough but also advantageous for tumorigenesis. Moreover, most APC truncations observed in colon tumors result in exclusion of the 3rd 20 a.a. repeat from the protein product [88, 92, 94]. Combining these mutation patterns defines a region between amino acids 1250- 1464 where most APC mutations are detected, named the mutation cluster region, MCR (figure 1.1) [95]. The same interdependence of both somatic *APC* mutations is also seen in sporadic cases of CRC, although it is not possible to determine the time sequence of these mutations [92, 94]. This apparent selective advantage of retaining the N-terminal half of APC suggests a dominant-negative effect of this truncated allele [35]. Other groups proposed the "just right" hypothesis for WNT signaling control as an explanation for this selective advantage of the MCR [92, 96]. This hypothesis suggests that truncation of the C-terminal half of APC increases WNT signaling to a submaximal level which is 'just right" for tumor development. Neither maximal upregulation of WNT signaling with mutations 5' to the MCR nor minimal upregulation of WNT signaling by mutations 3' to the MCR confer the same tumorigenic advantage to the transformed cells [96].

Nieuwenhuis and Vasen performed a meta-analysis for genotype-phenotype correlation in FAP patients [97]. They found that germline mutations in the MCR have the most severe intestinal polyposis phenotype, developing over 5000 colonic polyps. Mutations 5' or 3' to the MCR were associated with an intermediate intestinal polyposis phenotype. Mutations that result in a truncation in APC after amino acid 1595 or before amino acid 157 were associated with an attenuated phenotype with development of few polyps [97]. Complete deletion of APC has been reported only rarely and results in an intermediate phenotype [23, 98]. As for the extra-colonic phenotypes, Nieuwenhuis and Vasen found that extra-colonic manifestations are fairly common in FAP patients (around 70%). CHRPE is the most frequent phenotype, and was correlated with APC truncation between amino acids 311- 1446. Desmoid tumors, on the other hand, were correlated with APC truncations 3' to the MCR, after amino acid 1400. Duodenal and gastric tumors were correlated with *APC* mutations downstream of codon 1395 and 564-1450 [97]. It is important to note that these genotype-phenotype correlations are not rigid or complete, which suggests roles for other genetic and environmental factors in tumor development [97, 99].

Control of APC levels in the cell

Although APC has important roles in development and cancer, control of the cellular APC level is not well-understood. APC is widely expressed in most tissues [20]. Using immunohistochemistry to detect APC, Smith and colleagues found that cells near the surface of normal colon mucosa stained more than those in the bottom of the crypts, which suggests that the APC level increases with cellular differentiation [28]. Jaiswal and Narayan studied 921-bps of APC promoter, including 649 bps 5' and 272 bps 3' to the

transcription start site. They characterized 3 E-boxes (A, B and M), a CAAT box, an OCT1-binding site and an AP2-binding site in the region 5' to the transcription start site. Using chloramphenicol acetyltransferase reporter (CAT) and electrophoresis mobility shift assays (EMSA), they found that the transcription factors upstream stimulating factor 1 and 2 (USF1 & USF2) bind to the 5' region of the *APC* cloned promoter area and stimulate the expression of the reporter gene [100].

Although not completely delineated, the cellular level of APC seems to be controlled at different levels. At the transcriptional level, *APC* mRNA are induced in response to the DNA damaging agent N-methyl-N-nitro-N-nitrosoguanidine (MNNG) in one colon cancer cell line [101]. This upregulation of *APC* transcription in response to DNA damage seems to occur through the P53 pathway. Interestingly, this induction was not detected in 2 other non-colon cancer cell lines [101, 102]. Another example of control of *APC* transcription was shown by the Nathke and Rocha groups. They showed that upregulation of hypoxia-induced factor 1α (HIF-1α) by growing cultured cells under hypoxic conditions, or through direct HIF-1α overexpression, reduces *APC* mRNA and protein levels. They also showed that HIF-1α binds to the *APC* promoter sequence [103]. Hypermethylation of the *APC* promoter has also been detected in many cancers including breast, endometrial, prostate, and parathyroid [104-107]. However, a direct relation between this promoter hypermethylation and expression of APC has not been established, and the significance of this promoter hypermethylation is not fully understood [105, 108, 109].

At the translation level, *APC* mRNA has been shown to interact with miRNAs and an RNA-binding protein to control APC translation. Overexpression of miR-135

family members miR-135a and miR-135b reduced the APC protein level in cultured cells [110]. miR-135a and miR-135b decrease the expression of a luciferase reporter construct containing the *APC* 3'UTR region. Interestingly, the same group also showed that both miR-135a and miR-135b are upregulated in many colon cancer cell lines [110]. Tao and Xu proposed that *APC* mRNA is a target for miR-27. They showed that miR-27 downregulation during osteoblast differentiation is correlated with reduction of β-catenin levels. Overexpression of miR-27 reduced transcription of a luciferase reporter that contains the *APC* 3'UTR region [111]. In addition, Spears and Neufeld have shown that APC and the RNA-binding protein MSI1 are in a double-negative feedback loop [112]. They and another group have independently shown that MSI1 is a WNT target gene and thus inhibited by APC [112, 113].The Neufeld lab has also shown that MSI inhibits *APC* mRNA translation, reducing APC protein level in the cell. Spears and Neufeld reasoned that this putative interaction could play a role in cell fate determination and in maintenance of the stem cell population [112].

At the protein level, Choi *et al.* have shown that expression of APC with ubiquitin protein (Ub) reduces the APC level in the cells. This reduction of APC level is inhibited by treating the cells with a specific proteasome inhibitor, which indicates that APC is degraded by the ubiquitin proteasome pathway [114]. Harris and Nelson found 2 slowly-migrating phosphorylated forms of APC in human umbilical vein endothelial cells (HUVECs). They showed that inhibiting proteasomes with MG132 results in accumulation of both forms, while inhibiting GSK3β and CK1 (both kinases components of the β-catenin destruction complex) results in disappearance solely of the slower migrating form. They found that both forms have different half-lives (3.5 hours for the

slower and 30 hours for the faster migrating forms), through inhibition of production of new APC via cyclohexamide (CHX) followed by Western blotting. They concluded that APC undergoes phosphorylation and destabilization by the β-catenin destruction complex [115].

Colon cancer, inflammation and APC

Chronic colitis is another major risk factor for CRC. Patients with an inflammatory bowel disease (IBD, ulcerative colitis, or Crohn's disease) have 4- 20 fold increased risk or suffering CRC relative to the general population [116]. This colitis-associated CRC is more aggressive than is sporadic CRC and is fatal in 50% of cases [4]. As with sporadic CRC, colitis-associated CRC develops from cells through accumulated mutations. However, the nature of the mutated genes and the order of their mutation are different [117-119]. The first step in most cases of both colitis-associated and sporadic colorectal carcinogenesis is upregulation of WNT signaling, but this results from different mechanisms [4]. In sporadic CRC, mutation of both *APC* alleles results in accumulation of β-catenin in the cell [7]. In colitis-associated CRC, several inflammatory pathways inhibit the β-catenin destruction complex or directly activate β-catenin, resulting in upregulation of WNT signaling [4]. Notably, mutations of APC are still detected in colitis-associated CRC, though at later stages of carcinogenesis [117-120]. Many inflammatory mediators have been linked to the pro-tumorigenic effects of inflammation in IBD [116]. These inflammatory mediators include IL-1α, IL-1β. TNF-α, IL-17, IL-6, IL-10, and EGF [116]. These mediators are secreted by inflammatory cells as well as by epithelial cells, and affect cellular survival, proliferation, apoptosis, and differentiation [4, 116]. Many inflammatory pathways converge to activate the NF-κB

pathway [4]; activation of the NF-κB pathway was detected in colonic mucosa from IBD patients [121]. In addition, activation of the NF-κB pathway was shown to increase proliferation and decrease apoptosis in CRC cell lines and mouse colon mucosa [121, 122]. Moreover, activation of NF-κB increases levels of total and nuclear β-catenin in colonic cells in mice, possibly through inhibition of the β-catenin destruction complex [122]. Inhibiting NF-κB using a specific peptide abolishes this activating effect on cellular β-catenin [122]. Other signaling pathways that are altered in colitis-mediated CRC include AKT, KRAS, BRAF, and TGF-β pathways [4]. Inflammation could also increase the rate of DNA mutations by increasing the production of reactive oxygen species (ROS) [123, 124]. In addition, chronic inflammation was correlated with histone modifications and DNA methylation [125]. The protumorigenic effect of chronic colitis has been also linked to prostaglandin (PG) formation through induction of cyclooxygenase-2 (COX-2) [126]. COX-2 is the rate-limiting step in PGE2 formation from arachidonic acid [125]. Through use of cultured cells and mouse models, PGE2 has been shown to promote WNT signaling, increase cellular proliferation, inhibit apoptosis, promote angiogenesis, and enhance metastasis [127-130].

Inflammation and WNT signaling are not completely independent factors. As mentioned above, inflammation can activate Wnt signaling through many pathways, including NF-κB and PGE2 [131]. In turn, COX-2 has been shown to be a WNT target [132, 133]. Moreover, it has been reported that *APC* mutations can alter retinoic acid metabolism to cause Wnt-independent up-regulation of COX-2 [134]. Upregulation of COX-2 is not merely restricted to colitis-associated CRC, but was also detected in sporadic cases of CRC [125]. Finally, NSAIDs that inhibit COX-2 activity reduce both

sporadic and colitis-associated CRC [135, 136]. These results suggest a role for inflammation, possibly caused by interaction with colonic bacterial flora, in sporadic cases of CRC [126, 134, 137].

Modeling colitis-associated CRC in mice

To facilitate studying colitis-associated CRC, the dextran sodium sulfate (DSS) rodent model was developed [138]. This model was described first in hamster, then was adapted to both rat and mouse [139]. In this model, colonic inflammation is induced by giving mice the resin DSS in a concentration of 1-4% in drinking water for 3- 7 days. Following DSS exposure, mice are given untreated water for 2-4 weeks. This cycle could be repeated up to four times [138].

It is thought that the DSS model closely resembles human ulcerative colitis both pathologically and molecularly [140]. Administration of DSS models the acute phase of the disease. The pathological changes seen during the first cycle in murine colons include loss of the crypt structure and ulceration, symptoms that are also seen in the acute phase of human disease [141]. Following the first cycle, mucosal regeneration, crypt branching and shortening, glandular disorder, and diarrhea are also seen; these also occur in the chronic phase of ulcerative colitis in humans. As with the human disease, mice treated with DSS also show an increased incidence of colonic tumors [141, 142]. The incidence of tumors in these mice varies based on the protocol of DSS treatment; the incidence is about 18% at age 120 days in Swiss mice treated with 4 cycles (7 days each) of 4% DSS [142]. More than half of the tumors that develop in DSS-treated mice are flat adenomas, similar to those seen in the human disease [142]. Some tumors in this model show malignant transformation [142]. Molecular changes in tumors from DSS-treated mice

also recapitulate those in colitis–associated colorectal carcinogenesis in human [118, 142].

To increase the incidence of colonic tumors in DSS-treated mice, several groups adopted administration of a mutagen with DSS [140]. The most commonly used mutagen is azoxymethane (AOM) [140]. A single intraperitoneal dose of AOM increases the incidence of colonic tumors in mice up to 100% [140]. Another advantage of using AOM is that it allows reduction of the DSS dose in mice, and decreases the mortality from DSS-associated acute colitis. Again, different groups use different regimens of AOM treatment: single or multiple doses of 7.4- 20 mg/kg, but a single dose of 7.4mg/Kg of AOM alone is insufficient to induce tumors in mice [140]. AOM induces O^6-methylguanine DNA adduct resulting in G→A transitions. β-catenin mutations in exon 3 are detected in most tumors from AOM-DSS-treated mice. These mutations are expected to prevent phosphorylation and targeting of β-catenin by the destruction complex, resulting in cellular accumulation and nuclear translocation of β-catenin, and upregulation of Wnt signaling [143, 144]. Another commonly detected mutation in tumors from AOM-DSS-treated mice is a gain-of-function (activating) mutation of *Kras* codon 12 [143]. As mentioned before, both WNT and RAS pathways are usually activated in human CRC [7].

Heat-shock response

Heat-shock response refers to an adaptive response to a sudden change in cellular environment (stress), which prevents cellular damage through induction of cellular heat shock proteins/ molecular chaperons/ stress proteins [145]. Although first described in response to heating, other stresses including oxidative and metabolic stresses also induce

the heat shock response. Molecular chaperones have many essential functions in the cell under both normal and stress conditions. They bind to unfolded proteins, assist them in refolding, prevent them from aggregating with themselves or with other proteins, and help to solublilize protein aggregates. Molecular chaperones bind to damaged proteins to sequester them, refold them, or target them for degradation if the damage is severe [146]. Some molecular chaperones also help in translocation of other proteins to different cellular compartment such as mitochondria [147]. These abilities explain why some heat-shock proteins are constitutively expressed, while others are induced in response to cellular stress. Heat-shock proteins are classified by their molecular weight, e.g. HSP70 and HSP90 are 70 and 90 kDa, respectively. Their activity is energy-dependent, obtained by hydrolyzing ATP which is accompanied by cycles of binding and releasing the client proteins to help refold them. Heat-shock proteins usually function in complexes with other proteins (co-chaperones) and help chaperones in their functions [145, 147].

Activation of heat-shock factor 1 (HSF1) induces the heat-shock response (figure 1.4) [148]. In cells under normal (non-stress) conditions, HSF1 is present mostly in a complex with other molecular chaperones, including HSP90 [149]. During stress, the damaged and unfolded proteins bind chaperones, resulting in release of HSF1 from the complex [149]. HSF1 has a nuclear localization signal that directs HSF1 to the nucleus where it undergoes phosphorylation and forms a homo-trimer. The HSF1 trimer binds to a specific DNA sequence (heat-shock element, HSE) in the promoters of some genes essential for responding to the stress, including those encoding the chaperone proteins themselves. Binding of HSF1 to HSE reactivates a paused RNA polymerase to transcribe these genes. When the stress subsides, molecular chaperones sequester HSF1 in the cytoplasm to stop

the stress response [148, 150]. Increasing the concentration of cellular unfolded proteins (e.g. by inhibiting proteasomal degradation) induces the heat shock response by this same unfolded-protein response mechanism [145].

Given their importance in cell survival, heat shock proteins are emerging drug targets [151]. HSP90 inhibitors have toxic effects on cancer cells; some, such as 17-AAG and 17-DMAG, are in clinical trials for treatment of patients with advanced malignancies [152]. Many cancer cells depend on molecular chaperones for folding onocoproteins, even those that are mutated, chimeric, or overexpressed, such as mutated P53 and BRCA1 [151]. Inhibition of HSP90 results in reduction in the level of these oncoproteins, and sensitizes these cells for other chemo and radio-therapies [153]. One advantage of this strategy is that many proteins in different oncogenic pathways are targeted at the same time, decreasing the chance of developing drug resistance [146, 151]. However, the wide range of HSP90 client proteins also makes these drugs very toxic for normal cells. In addition, inhibiting HSP90 results in release of HSF1 from the cytoplasmic complex, which then induces expression of heat shock proteins including HSP90 itself (figure 1.4) [149]. This feature reduces the efficacy of HSP90 inhibitors as anticancer agents [154]. On the other hand, as molecular chaperones are important in assisting with protein folding and preventing protein aggregation, induction of heat-shock response has been proposed to be useful for the treatment of neurodegenerative disorders characterized by accumulation of toxic protein aggregates [155]. Interestingly, HSP90 inhibitors have been used to induce heat shock response in these patients. Again, these agents are toxic and are not completely safe [156].

The long-term goal of the Neufeld lab is to understand homeostasis of intestinal epithelial cells, and how disruption of the normal control mechanisms leads to intestinal tumor formation. In the Neufeld lab, we use different models, including colon cancer cell lines and different mouse models, to study APC. In chapter 2, I review different germline and conditional Apc-rodent models, and discuss the impact of these models on understanding intestinal tumorigenicity and different aspects of APC biology. Dr. Neufeld has described the localization of APC to the nucleus. She characterized the 2 nuclear NLS and 3 NES domains of APC. In order to understand the physiologic role of nuclear APC within a whole organism, the Neufeld lab has made a new mouse model in which nuclear APC is compromised via inactivating knock-in mutations in both Apc NLS domains ($Apc^{mNLS/mNLS}$ mice). In chapter 3, I show that $Apc^{mNLS/mNLS}$ mice have upregulated Wnt signaling and increased proliferation in intestinal epithelial cells, relative to signaling and proliferation in wild-type mice. I also show that $Apc^{Min/+}$ mice, the most widely-utilized mouse model with a germline Apc mutation causing protein truncation, develop more and larger intestinal tumors when they also harbor the Apc^{mNLS} mutant allele ($Apc^{mNLS/Min}$). In chapter 4, I show that $Apc^{mNLS/mNLS}$ mice have a higher susceptibility of colitis-associated colon tumors, through use of the AOM-DSS model. In chapter 5, I show data characterizing $Apc^{mNLS/Min}$ mice including testing LOH in intestinal polyps and some extra-intestinal phenotypes in these mice. In chapter 6, I describe characterization of and screening for polymorphic loci in the promoter of *Mom-1* (*Pla2ga2)* gene in our mouse colonies. I show that this polymorphic allele is associated with increase expression of *Pla2ga2* and prolonged survival in the Apc mouse model $Apc^{1322T/+}$. The Neufeld lab has also a long interest in studying different mechanisms

controlling cellular APC levels, and how control of these levels could affect intestinal tumorigenesis. In chapter 7, I show that induction of the heat-shock response increases the APC level in colon cancer cell lines and in mouse intestinal epithelial cells. I show also the results of a series of experiments in different mouse models testing the effects of the induction of the heat-shock response on intestinal tumorigenesis. The results suggest that induction of the heat-shock response using the novel non-toxic small molecule KU-32 and the commercially available drug 17-AAG changes intestinal tumor number, size and regional distribution in mice. In Chapter 8, I summarize the findings of my studies and their implications for our knowledge of APC and colon cancer and discuss some future directions of these projects.

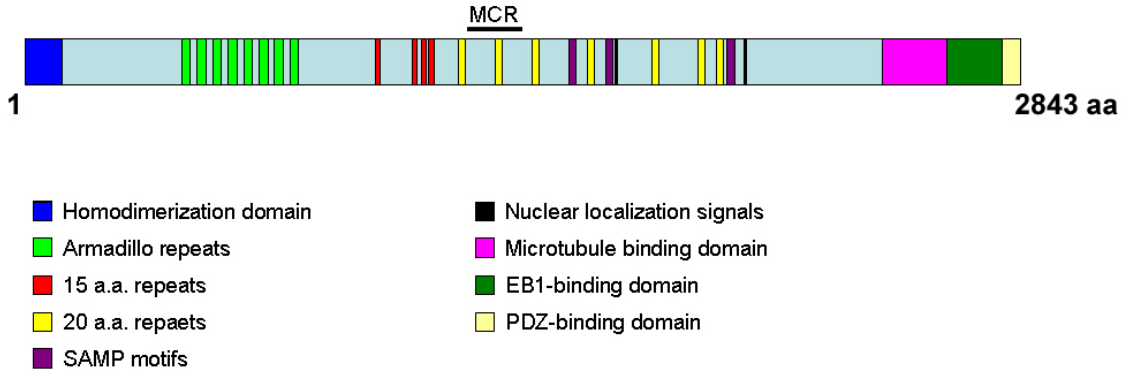

Figure 1.1: APC structural domains. MCR refers to the mutation cluster region.

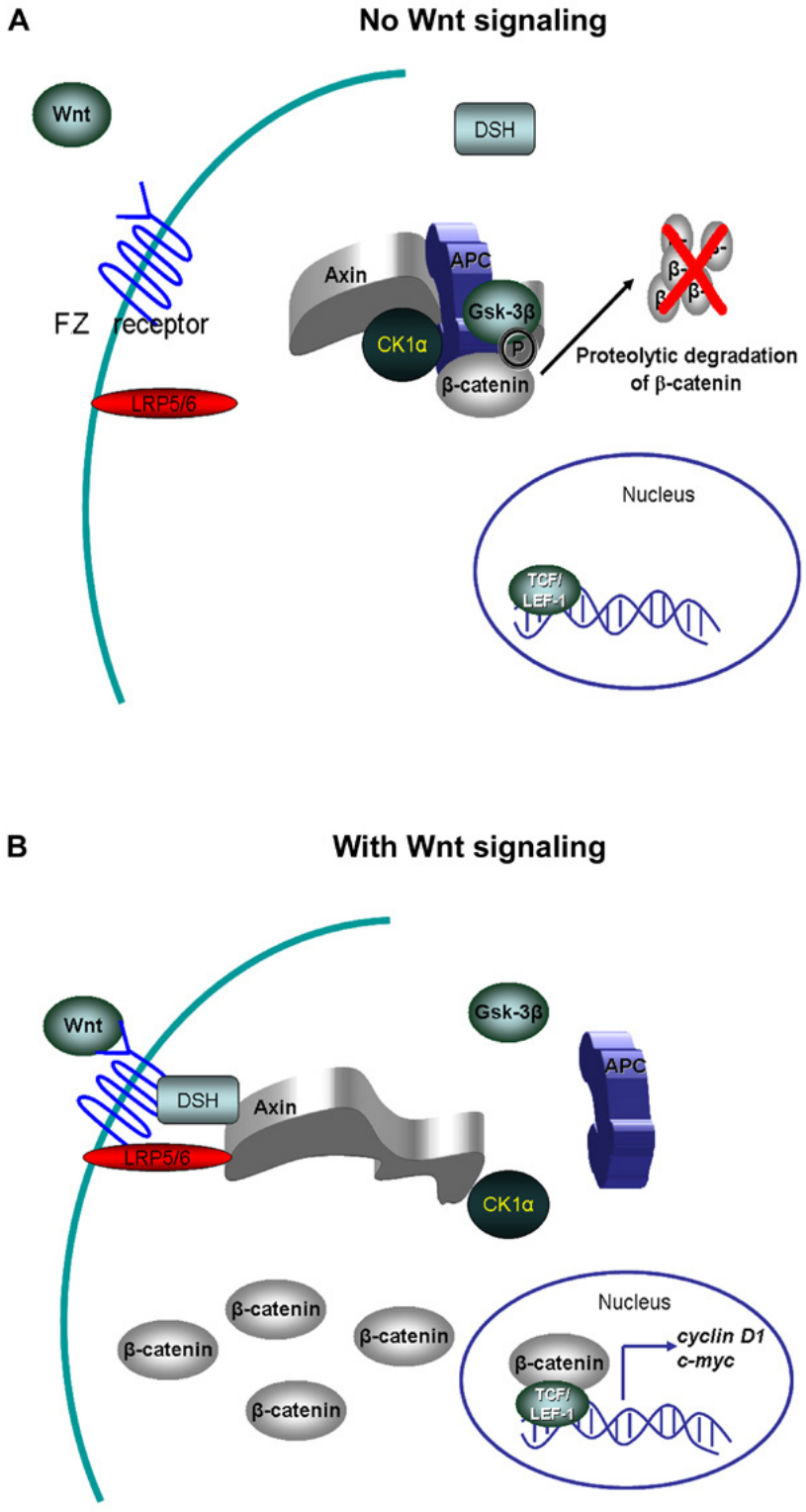
A
No Wnt signaling
Wnt
DSH
FZ receptor
LRP5/6
Axin
APC
Gsk-3β
CK1α
P
β-catenin
Proteolytic degradation of β-catenin
Nucleus
TCF/ LEF-1
B
With Wnt signaling
Wnt
DSH
LRP5/6
Axin
Gsk-3β
APC
CK1α
β-catenin
β-catenin
β-catenin
β-catenin
Nucleus
β-catenin
cyclin D1
c-myc
TCF/ LEF-1

Figure 1.2: Wnt signaling. (A) In the absence of Wnt, the β-catenin destruction complex is formed. This β-catenin destruction complex phosphorylates β-catenin and targets it for proteasomal degradation. **(B)** Wnt ligand binds to Wnt receptor Frizzled (FZ), recruiting LRP5/6 Disheveled (DSH) and Axin, inhibiting β-catenin destruction complex β-catenin. β-catenin accumulates in the cell and translocates to the nucleus where it works as a transcription co-activator with TCF/LEF, inducing expression of Wnt target genes such as CyclinD1 and cMyc. (Adapted from Neufeld)

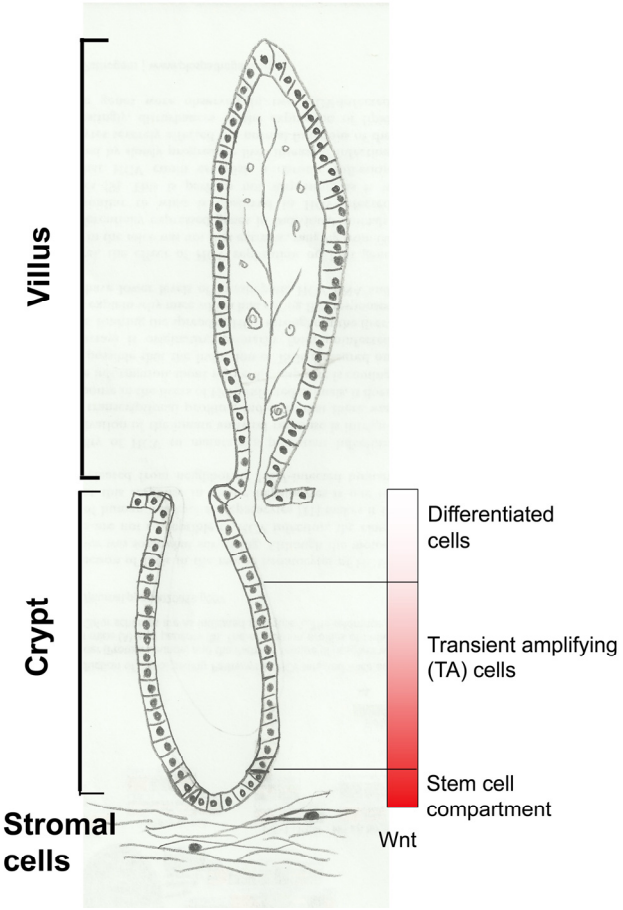

Figure 1.3: Role of Wnt signaling in maintaining intestinal structure. A schematic diagram for the structure of the small intestine shows that the small intestine is made of sac-like invaginations (crypts) and finger-like projections (villi). Stromal cells at the base of the crypt base secrete Wnt, maintaining a gradient concentration of Wnt along the length of the crypt. At the base of the Crypt with the highest Wnt signaling, stem cells divide to maintain stem cell population and also give rise to transient amplifying (TA) cells. TA cells divide and migrate along the crypt until the upper 1/3 of the crypt (low Wnt concentration) where cells start to differentiate into different intestinal types. The structure of the colon is very similar except that colon epithelium does not have villus structures.

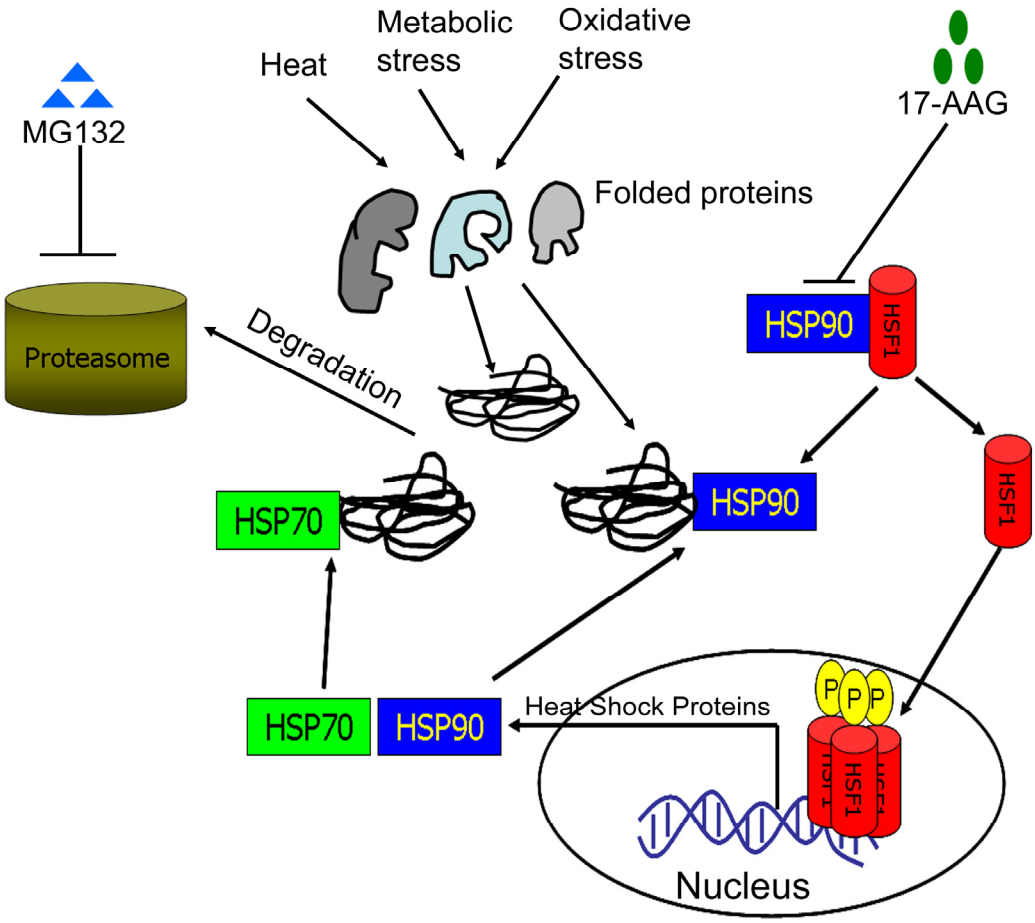

Figure 1.4: Induction of the heat-shock response. Under non-stress conditions, heat-shock factor 1 (HSF1) is sequestered in a cytoplasmic complex containing heat-shock proteins, including heat shock protein 90 (HSP90). When the cells are exposed to thermal, metabolic, oxidative, or other stress, HSP90 binds to damaged-unfolded proteins, helps to refold them and prevents aggregate formation. Binding HSP90 to damaged proteins results in releasing HSF1 from the complex. HSF1 goes to the nucleus, where it forms a trimer and undergoes phosphorylation. HSF1 trimer works as a transcription factor, inducing the transcription of many proteins including heat-shock proteins (heat-shock response). Inhibiting HSP90 via 17- AAG, or increasing unfolded proteins via inhibiting the proteasome, also releases HSF1 and induces the heat-shock response.

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Chapter 2

More than two decades of Apc modeling in rodents

Abstract

Mutation in the tumor suppressor gene Adenomatous polyposis coli (*APC*) is the initiating step in most colon cancers. To facilitate studying APC functions in intestinal development and tumorigenesis, more than a dozen models have been generated including mouse, rat, zebrafish, *Drosophila,* and *C.elegans*. This review summarizes the phenotypes of 35 different rodent Apc models and knowledge gained using these models to study *Apc*, with particular concentration on the most recently developed models. We also discuss variation in phenotypes among different models and the correlation between genotype and phenotype.

APC mouse models

For over a century, mice have been used to study human diseases and to test potential drugs and toxins. Among vertebrates, mice have the advantage of relatively short life cycles, large litter sizes, ease of care, and conservation with humans of many physiological and pathological processes [1]. The APC protein is well-conserved between human and mouse, with 87.9% amino acid identity and 91.9% similarity [2]. In addition, all characterized motifs in human APC are conserved in murine Apc [2]. This structural conservation predicts functional conservation, and makes the mouse a good model to study the physiological role of APC in a whole organism. Mice allow experimental dissection of physio-pathological roles of APC in intestinal homeostasis and vertebrate development, which are less tractable in other models [3].

APC mouse models can be divided into 2 broad categories. In the first category, mice have a germline mutation in *Apc* that results in protein truncation or alteration or reduced expression of Apc protein in all tissues (figure 2.1). In the latter category, *Apc* gene mutation is induced only in specific mouse tissues at a particular stage of development. The phenotypes of the various *Apc* mutant mice differ, depending on many factors that will be discussed below.

Apc$^{Min/+}$

The Multiple intestinal neoplasia (Min) mouse was first identified in an ethylnitrosourea (ENU) mutagenesis screen, [4] and is the best characterized mouse model with a germline *Apc* mutation [2]. Apc^{Min} mice have a nonsense mutation that results in truncation of Apc protein at codon 851 [2]. Since its first description in 1990, the Apc^{Min} model has been used extensively to study Apc functions in suppression of intestinal tumorigenesis and to investigate tumor prevention strategies. Mice homozygous for *Apc^{Min}* die early in embryonic development, but Apc$^{Min/+}$ mice display both intestinal and extra-intestinal phenotypes [2, 4-6]. In the C57Bl/6J background, Apc$^{Min/+}$ mice live for ~120 days and typically develop between 20-100 polyps in their gastrointestinal tract [4]. The intestinal polyp phenotype is 100% penetrant. However, the polyp number can vary between one and >200 polyps per mouse. Differences in diet, flora, genetic background, and genetic modifiers might account for this variability [7, 8]. The vast majority of these polyps are in the small intestine, with a few developing in the colon and even fewer in the stomach [4, 5]. Histologically, most tumors in Apc$^{Min/+}$ mice are benign adenomas: polypoidal, sessile, or papillary in nature, with limited dysplasia and atypia. Although these polyps can reach the size of 8 mm in diameter, malignant changes are not

typically seen. However, in old $Apc^{Min/+}$ mice, polyps express molecular markers of invasiveness seen in malignant tumors, [9] and areas with limited invasion and carcinoma *in situ* have been observed [4]. In addition, $Apc^{Min/+}$ mice in hybrid genetic backgrounds (F1 B6 X BR6 and B6 X SWR) live longer and develop fewer polyps than do mice in the B6 background, but show malignant changes and local metastasis to lymph nodes [10]. Together, these data suggest that the short life span of $Apc^{Min/+}$ mice limits the accumulation of other genetic mutations in intestinal tumors that are required for progression to invasive carcinoma [10].

LOH in polyps of $Apc^{Min/+}$ mice

In both $Apc^{Min/+}$ mice and in FAP patients, mutation of the wild-type *Apc* allele is required for adenoma formation. However, the nature and predicted mechanism of the second mutation is different in $Apc^{Min/+}$ mice and in human FAP patients. Luongo *et al.* [11] from the laboratory that developed the Apc^{Min} model, bred a $Apc^{Min/+}$ B6 strain mouse with a wild-type AKR strain mouse. Use of PCR with incorporation of P^{32}-labeled thymine revealed a reduction in the ratio of the frequency of the AKR Apc^{+} allele to the frequency of the B6 Apc^{Min} allele in all tested polyps. This result suggests that LOH is required for polyp formation. Furthermore, adenomas had no change in the copy number of 6 other polymorphic markers on B6 chromosome 18, the location of the mouse *Apc* gene, and thus it was concluded that loss of the whole AKR chromosome occurred by means of mitotic non-disjunction, without duplication of the B6 Apc^{Min} allele [11].

Although the occurrence of LOH in polyps from $Apc^{Min/+}$ mice was confirmed later in several other studies, the mechanism of LOH suggested from this initial study was challenged by subsequent data. In 2002, Haigis *et al*., from the same laboratory, used

FISH to show diploidy of chromosome 18 and of the *Apc* locus in polyps from B6 $Apc^{Min/+}$ mice [12]. There are two potential explanations for these contradictory results. First, in the initial study, it was assumed that polyps from B6 X AKR $Apc^{Min/+}$ mice develop by the same mechanism as in B6 $Apc^{Min/+}$ mice. This might not be the case. Second, technical limitations of PCR quantification at the time of the first study should inform cautious interpretation of the results.

Chromosome 18 is acrocentric in mice, and a single recombination event near the centromere will appear as a complete loss and duplication of the whole chromosome resulting from a mitotic non-disjunction event [12]. To address this issue, Haigis *et al.* bred $Apc^{Min/+}$ mice with a mouse that has a fusion of 2 acrocentric chromosomes, 7 and 18 (Robertsonian translocation of chromosome 7 over 18, *Rb9*, see figure 2.2). They used a natural mutation in the *Tyr* gene in a heterozygous state on the chromosome 7 arm of the *Rb9* translocation chromosome as a marker for mitotic events that involve the whole chromosome versus one arm of the rearranged chromosome. They assessed LOH of *Apc* on one arm (chromosome 18) and LOH of the *Tyr* gene on the other arm (chromosome 7) of the fused chromosome (*Rb9*) in polyps that developed in these mice. They found no evidence for loss of both arms of the rearranged *Rb9* chromosome in 77% of tested polyps from these mice. In the remaining 23% of the polyps, they could not differentiate between loss and duplication of the whole chromosome versus recombination involving both arms of the rearranged chromosome as the underlying mechanism of polyp initiation (figure 2.2) [12]. In 2003, the same group showed that the presence of the *Rb9* chromosome is associated with reduced tumor burden in $Apc^{Min/+}$ mice when placed in trans, cis, or in homozygous distribution with the Apc^{Min} allele (figure 2.2C) [13]. They

concluded that somatic recombination (rather than complete deletion and reduplication of the whole chromosome) is the mechanism of polyp formation in these mice. In this case the reduction of mitotic recombination that is seen in *Rb9* mice would have been expected to reduce LOH and the intestinal adenoma burden [13]. However, because translocation of the *Rb9* chromosome could also affect chromosomal segregation, complete loss and duplication of chromosome 18 in adenomas from $Apc^{Min/+}$ mice without a *Rb9* chromosome couldn't be ruled out.

More recent evidence supports loss and duplication of the entire chromosome 18 as the mechanism of LOH in polyps from $Apc^{Min/+}$ mice [14]. Mutation of one allele of *Atp5a1,* also on chromosome 18, decreases the number of polyps in $Apc^{Min/+}$ mice if the mutant *Atp5a1* allelle lies on the same chromosome with the Apc^{Min} allele. Furthermore, distribution and histopathological characteristics of polyps are different in mice with *Apc* and *Atp5a1* mutations in cis distribution, but not in trans, relative to polyps from $Apc^{Min/+}$ mice with the wild-type *Atp5a1* allele. Because mutation of both *Atp5a1* alleles is lethal to cells, a cell that loses chromosome 18 carrying the wild-type *Atp5a1* allele should be eliminated and thus, complete loss with or without subsequent duplication of chromosome 18 would be prevented if mutations in the *Apc* and *Atp5a1* genes are on the same chromosome. In contrast, there would not be such a negative selection against loss and reduplication of the whole chromosome if mutated *Apc* and *Atp5a1* are in trans distribution. Since polyps that formed in the latter situation were indistinguishable from those that developed in $Apc^{Min/+}$ mice with wild-type *Atp5a1* alleles, it was concluded that loss and duplication of chromosome 18 is the likely mechanism of LOH in polyps from $Apc^{Min/+}$ mice [14].

Analysis of these data suggests that loss of the Apc^{+} allele with locus diploidy is the mechanism of the second *Apc* "hit" needed for the development of most polyps from $Apc^{Min/+}$ mice in the B6 background. Whether this LOH is the result of somatic recombination or complete loss and duplication of chromosome 18 is still not completely resolved, however. One potential challenge in characterizing the underlying mechanism is that, because chromosome 18 is acrocentric, a single somatic recombination proximal to the Apc locus will be difficult to distinguish from complete loss and reduplication of the whole chromosome [12].

Mammary gland tumors

In addition to intestinal tumors, $Apc^{Min/+}$ mice also develop mammary tumors, but at a much lower penetrance (5%) and at a relatively older age (16 ±3.5 weeks) [6]. Histologically, these tumors are usually invasive in nature, with areas of adenoacanthoma and adenocarcinoma [6]. $Apc^{Min/+}$ mice treated with the mutagen ENU, exposed to X-rays, or with a mutation in the DNA repair gene *Myh,* have increased mammary tumor incidence without affecting tumor morphology, which indicates that *Apc* mutation alone is not sufficient for mammary tumorigenesis [15, 16]. Expression of a stabilized form of β-catenin in mammary epithelium results in the development of tumors in mice with wild-type *Apc.* This is consistent with a role for *Apc* in inhibiting mammary tumorigenesis via antagonizing the Wnt signaling pathway [6, 17, 18]. Although FAP patients also have an increased risk of tumors outside the gastrointestinal tract, including desmoid tumors, mandibular osteomas, and retinal dysplasias, these patients do not show increased susceptibility to breast cancer [19]. In addition, the pathology of the mammary tumors seen in $Apc^{Min/+}$ mice is not typically seen in humans [6]. However, mutation of

APC and methylation of its promoter have been detected in up to 70% of cases of breast cancer in humans, which indicates a role for APC in suppression of mammary neoplasia [20-22].

Extra-intestinal phenotypes

Although intestinal polyposis is the dominant feature in $Apc^{Min/+}$ mice, these mice show additional changes in other tissues [4, 23]. There is no evidences that LOH is necessary for the extra-intestinal phenotypes of $Apc^{Min/+}$ mice, which suggests that *Apc* haplo-insufficiency is the underlying mechanism. Anemia was the first extra-intestinal phenotype to be described in $Apc^{Min/+}$ mice and was used to predict intestinal polyposis before the establishment of Apc^{Min} genotyping [4]. Although the exact pathogenesis is not completely understood, anemia in $Apc^{Min/+}$ mice is microcytic-hypochromic, consistent with chronic blood loss from intestinal lesions as the underlying cause [4]. Old $Apc^{Min/+}$ mice also develop large spleens with enhanced splenic hematopoiesis, which suggests that larger spleens might be an extra-medullary compensatory response to anemia. But because larger spleens and anemia are not always correlated in $Apc^{Min/+}$ mice, a different mechanism for large spleen development might be at play [23]. Old $Apc^{Min/+}$ mice can also develop myelodysplastic disease, with increased formation of myeloid, granulocytic, and erythroid colonies in the spleen [24, 25]. Other hematological changes seen in $Apc^{Min/+}$ mice include rapid thymus regression, depletion of splenic natural killer (NK) cells, and loss of B-lymphocyte progenitors in spleen and bone marrow [26]. Using bone marrow transplantation, Coletta *et al.* showed that these changes result from disruption of the bone marrow micro-environment in $Apc^{Min/+}$ mice [26]. Lane *et al*. suggested that

gradual loss of the quiescent hematopoietic stem cells occurs in $Apc^{Min/+}$ mice, and might reflect haplo-insufficiency for the Apc^{Min} allele [25].

Other extra-intestinal phenotypes include increased number of degenerated and undeveloped ovarian follicles, and under-developed testicular seminiferous tubules [23]. The cause of these gonadal changes in $Apc^{Min/+}$ mice is not known. However, conditional truncating mutations of *Apc* in testicular Sertoli cells result in premature germ-cell loss and the absence both of Sertoli cell apical extensions and of the blood-testis barrier. These changes were not recapitulated by activating mutations in β-catenin consistent with a Wnt-independent function of Apc in Sertoli cells [27]. Prosperi *et al.* described disruption and involution of mammary glandular structures in $Apc^{Min/+}$ pregnant females. They correlated these changes with altered proliferation, increased apoptosis, and interrupted epithelial integrity and polarization in mammary epithelial cells. They did not observe changes in transcription of Wnt targets or in nuclear localization of β-catenin. They concluded that these mammary gland phenotypes represent a Wnt-independent role for Apc [28]. In addition, at 15 weeks, $Apc^{Min/+}$ mice display a change in their serum lipid profile called dyslipidemia, with increased serum levels of triacylglycerol, cholesterol, and free fatty acids [29-32]. The exact cause of this dyslipidemia is not understood, but hyperlipidemia has been correlated with the activity and level of the lipid regulatory nuclear receptors PPAR α, β and γ [30, 31, 33]. Treatment of $Apc^{Min/+}$ mice with the nonsteroidal anti-inflammatory agent indomethacin both decreased polyp number and improved dyslipidemia in $Apc^{Min/+}$ mice [34]. These extra-intestinal phenotypes indicate that Apc has roles not only in intestinal epithelial cells, but also in development and maintenance of other tissues.

Use of the Apc^{Min} model to test the effect of environment, genetic alterations, and drugs on tumor formation

In addition to its use as a model of FAP, $Apc^{Min/+}$ mice have been used extensively as a tumor susceptibility model to test the effect of environmental factors, mutations in other genes, and drugs on intestinal tumorigenesis. Such studies have increased our understanding of both intestinal tumorigenesis and cancer biology in general. Details of these studies are summarized in several excellent reviews [3, 7, 8, 35].

Modifiers of Min *(Mom)*

Studying phenotypic variation in model organisms of different genetic backgrounds is a powerful tool to elucidate other genes contributing to the phenotype. These genes are classically termed modifiers [35]. Several modifiers have been described that can enhance or attenuate intestinal polyposis in $Apc^{Min/+}$ mice and are called *Modifiers of Min* (*Mom*) [7, 35]. The first and most characterized modifier locus is *Mom-1* (*Pla2g2a* gene) which decreases polyp multiplicity in some mouse strains [36, 37] (see chapter 6) [14, 36]. A detailed discussion of these modifiers and the mechanism of action could be found elsewhere [35].

Mouse models expressing truncated Apc protein longer than Apc^{Min}

APC is a large multi-domain protein that has been implicated in many cellular activities in addition to its role in down-regulating Wnt signaling. Most APC domains implicated in targeting β-catenin for degradation are in the middle region of APC. However, most tumor-associated mutations in *APC* result in loss of the C-terminal regions of APC [38, 39]. Interaction of C-terminal APC regions with DNA and with microtubules has been proposed to contribute to tumor suppression [40, 41]. Disruption

of the interaction between APC and microtubules affects spindle formation and mitosis in colon cancer cell lines and in intestinal epithelial cells in $Apc^{Min/+}$ mice [42]. In addition, $Apc^{Min/Min}$ embryonic stem (ES) cells show chromosomal instability (CIN) [43]. These observations led to the proposal that loss of the C-terminal domains of Apc promotes intestinal tumorigenesis through induction of CIN [43-45]. Three mouse models with truncations of the C-terminal third of Apc have been generated; Apc^{1638N}, Apc^{1638T}, and Apc^{1572T}. The Apc^{1638N} mouse was made in 1994 by anti-sense insertion of a neomycin-resistance gene at *Apc* codon 1660 resulting in a nonsense mutation at codon 1638 [46]. $Apc^{1638N/1638N}$ mice die as embryos. $Apc^{1638N/+}$ mice develop intestinal polyps, but the number of polyps (less than 10) is very small compared to the number in $Apc^{Min/+}$ mice, and polyp distribution is also different (gastric and colonic). Intestinal tumors in $Apc^{1638N/+}$ mice are also invasive, with distant metastasis in the liver detected in one mouse. Because these mice live longer than do $Apc^{Min/+}$mice, the invasive phenotype could reflect tumor progression over time. Intestinal tumorigenesis is enhanced in this Apc mouse model with mutation in other tumor suppressor genes [47-52]. LOH via loss of the entire chromosome 18 was suspected in most tumors from Apc^{1638N} mice [53]. Haigis *et al.* showed, however, that the Apc^{+} allele is maintained in most polyps from $Apc^{1638N/+}$ mice, which suggests the wild-type *Apc* allele is inactivated or silenced [54]. Desmoid tumors and cutaneous cysts develop in 100% of the $Apc^{1638N/+}$ mice [55]. In humans, desmoid tumors occur in FAP patients [56] and also in patients with an attenuated form of FAP (AFAP) resulting from germ-line mutations in the 3' regions of *APC* [57]. AFAP patients develop only a few polyps, mainly in the duodenum [57-59].

It could be argued that the Apc^{1638N} mouse is not the best model of AFAP, since only full-length Apc and not the truncated protein has been detected in these mice [46]. The explanation for this might be technical in nature. The antibiotic selection cassette used to generate the Apc^{1638N} mice is inserted in reverse orientation. Production of an antisense *Apc* transcript might lead to translation inhibition of truncated Apc in Apc^{1638N} could therefore be considered an essentially null allele [55, 60, 61].

The Apc^{1638T} mouse was generated to overcome this technical issue. With the antibiotic-resistance gene (hygromycin) inserted in the same orientation as the *Apc* gene, the expression of truncated Apc could be detected. One surprising finding is that Apc$^{1638T/1638T}$ mice are viable, whereas Apc$^{1638N/1638T}$ and Apc$^{Min/1638T}$ are not. Apc$^{1368T/1638T}$ mice have only slightly elevated Wnt signaling and do not develop intestinal or extra-intestinal tumors. Instead, these mice display post-natal growth retardation, nipple-associated cutaneous cysts, and lack preputial glands. The significance of these phenotypes is not well-understood, but could indicate a role for the C-terminal region of Apc in development. The Apc^{1638T} protein retains all 15 a.a. repeats, 1 SAMP motif, and 3 of the 20-a.a. repeats, but lacks 2 of the SAMP motifs and 4 of the 20-a.a. repeats [60]. Perhaps the remaining functions of the truncated *Apc^{1638T}* allele are dose-dependent, and thus the *Apc^{1368T}* allele is haplo-insufficient for β-catenin regulation.

More recently, the Fodde group developed the Apc^{1572T} mouse model by deleting the first SAMP repeat remaining in the Apc^{1638T} mouse [62]. The truncated Apc protein in Apc$^{1572T/+}$ mice is only 66 amino acids shorter than Apc from Apc^{1638T} mice. However, the phenotypes of these two mouse models are very different. Unlike *Apc^{1638T}*, *Apc^{1572T}* germ-line homozygozity is incompatible with viability. One remarkable feature of

$Apc^{1572T/+}$ mice in a B6 background is that they develop no intestinal tumors, but instead develop invasive mammary tumors that can even metastasize to the lungs. While mammary tumor morphology and LOH of the *Apc* allele are similar in both $Apc^{1572T/+}$ and $Apc^{Min/+}$ mice, the incidence of mammary tumors is much higher in $Apc^{1572T/+}$ mice; 100% in virgin females and 30% in males compared to only 5% in $Apc^{Min/+}$ females. β-catenin activity, as assessed by means of a TOPFLASH reporter assay, is higher in $Apc^{1572T/1572T}$ ES cells than in wild-type or $Apc^{1638T/1638T}$ ES cells, but lower than in $Apc^{1638N/1638N}$ ES cells. $Apc^{1572T/+}$ mice do develop intestinal polyps if they also have a $Smad4^{Sad}$ allele, which results in defective TGF-β signaling [62]. Because the TGF-β pathway inhibits Wnt signaling [63], the authors propose that in order to develop, a mammary tumor needs a certain level of Wnt signaling which is provided by the Apc^{1572T} allele. Increased Wnt signaling resulting from reduced TGFβ signal, or from a second mutant *Apc* allele, promotes intestinal polyp formation [62]. Although this model might explain the development of intestinal polyps in mice heterozygous for both Apc^{1572T} and $Smad4^{Sad}$ [64], it does not explain the high penetrance of mammary tumors in these mice, given the low penetrance of mammary tumors in other models with higher Wnt signaling.

In conclusion, data collected from these three mouse models implicated the C-terminal portion of Apc in control of mammary tumorigenesis and development (Apc^{1572T} and Apc^{1638T} mice). However, these models provide no direct evidence for a role of the Apc C-terminal region in suppression of intestinal tumorigenesis. Because Apc^{1638N} is virtually a null allele, intestinal polyps developing in these mice do not support or refute a specific role for the Apc C-terminus in polyp suppression.

Apc^{1309} and $Apc^{1322T/+}$ mice

Although $Apc^{Min/+}$ mice have been used to model *APC* mutation in humans, similarly sized APC truncations are uncommon in both inherited and sporadic human colon cancers. Mutations in *APC* associated with colon cancer typically truncate the C-terminal half of the protein, leaving the first 20-amino acid (20-a.a.) repeat intact in at least one *APC* allele [65]. As this 20-a.a. repeat can bind to β-catenin, one would predict differences in cells expressing shorter Apc truncations, such as Apc^{Min}, and cells with longer APC (as in human CRC) [66, 67]. The Apc^{1309} and Apc^{1322T} mouse models were generated to express truncated Apc that retains the first 20-a.a. repeat [65, 68, 69]. The Apc^{1309} mouse model was generated by the Noda group in the mid-1990s, while the Apc^{1322T} mouse model was made in 2009 by the Tomlinson group. As with $Apc^{Min/+}$, both $Apc^{1309/+}$ and $Apc^{1322T/+}$ mice develop polyps mainly in the small intestine, but these polyps are more proximal than those from $Apc^{Min/+}$ mice. Measuring the amounts of transcripts of Wnt target genes, Lewis *et al.* showed that Wnt target gene expression is lower in polyps from $Apc^{1322T/+}$ mice than in polyps from $Apc^{Min/+}$ mice, as expected since the Apc^{1322T} protein include the first 20-a.a. repeat[70]. However, $Apc^{1322T/+}$ mice develop more polyps (> 200 polyps by 12 weeks) and have more intestinal stem cells than do $Apc^{Min/+}$ mice [69]. These results support the "just right" hypothesis that predicts that inclusion of the first 20-a.a. repeat in truncated APC proteins will result in only slight elevation of Wnt signaling, which is more advantageous to tumor growth than is elevation of Wnt signaling to its highest possible level [67]. As to extra-intestinal phenotypes, $Apc^{1322T/+}$ mice develop anemia and large spleens, similar to $Apc^{Min/+}$ mice [69]. $Apc^{1309/+}$ mice develop an average of ~ 35 intestinal tumors, mainly in the small intestine at the age of 12-14 weeks, and have hyperlipidemia that develops at an even

earlier age than in $Apc^{Min/+}$ mice [29, 68]. Potential explanations for this large discrepancy in polyp number between mouse models that differ in truncated Apc length by only 13 amino acids include the influence of environmental factors, genetic background, and experimental procedures, described in more detail later.

Mouse models expressing truncated Apc protein shorter than Apc^{Min}

Seven mouse models with mutations upstream to that found in Apc^{Min} have been described. $Apc^{\Delta 242}$ [71], $Apc^{\Delta 474}$ [72], and $Apc^{\Delta 716}$ [73-75] mice have *Apc* truncation mutations at codons, 242, 474, and 716, respectively, while $Apc^{\Delta 580}$ [76], Apc^{580D} [77], and $Apc^{\Delta 14}$ [78] mice each have a deletion of exon 14, resulting in a frameshift and a nonsense mutation at codon 580. $Apc^{\Delta 15}$ mice have a deletion of the last *Apc* exon, including the 3'UTR region [79]. These seven mouse models share many phenotypes with $Apc^{Min/+}$ mice, including embryonic lethality in the homozygous state, and in heterozygous mice, development of anemia and intestinal polyps predominantly in the small intestine that are indistinguishable at the microscopic level [71-79]. Although polyp number varies between these seven models (Table 2.1), in most cases, direct comparative studies have not been performed. Mammary tumors have been reported for 14.3% of $Apc^{\Delta 580}$, 18.5% of $Apc^{\Delta 474}$, and 9% of $Apc^{\Delta 14}$ mice [72, 76, 78]. Is the phenotypic variation in polyp number in these mouse models due to the progressive deletion of particular Apc domains (see figure 2.1). The $Apc^{\Delta 716}$ protein is 134 a.a. shorter than the Apc^{Min} protein, and only lacks a C-terminal portion of the armadillo repeat domain. Although the three-fold increase in polyp number seen in $Apc^{\Delta 716/+}$ mice compared to $Apc^{Min/+}$ mice might result from interruption of the armadillo repeat domain, $Apc^{\Delta 242/+}$ mice, which have a truncating Apc mutation that eliminates the entire armadillo repeat

domain, develop only twice the number of polyps as do $Apc^{Min/+}$mice. Furthermore, $Apc^{\Delta 580/+}$, $Apc^{580D/+}$, $Apc^{\Delta 14/+}$ and $Apc^{\Delta 474/+}$ mice, which have truncating mutations in the middle of the armadillo repeat domain, have reported intestinal polyp numbers similar to that seen in $Apc^{Min/+}$ mice (Table 2.1).

Table 2.1: intestinal phenotypes in mice with truncated Apc longer than Apc^{Min}

Mouse model	Polyp number	$Apc^{Min/+}$ Polyp number*	Notes	Ref
$Apc^{\Delta 716}$	256±55	1/3 of those in $Apc^{\Delta 716}$		[75]
$Apc^{\Delta 474}$	123±9.6		No comparative data to Apc^{Min} mice	[72]
$Apc^{\Delta 242}$	177±30	106±28		[71]
$Apc^{\Delta 14}$	36±29	34±18	Different distribution than $Apc^{Min/+}$, number of polyps increases in germ-free environment	[78]
$Apc^{\Delta 15/+}$	184.7	-	Tumors are mainly in the ileum, no comparative data to Apc^{Min} mice	[79]
$Apc^{\Delta 580}$	120±37	-	No full-length or truncated Apc proteins were detected the polyps	[76]

***** included in the same study

Complete deletion of Apc

The majority of *APC* mutations seen in CRCs fall into a region referred to as the mutation cluster region (MCR), and result in truncation of the C-terminal half of APC [80]. Complete deletion of APC has been reported in FAP syndrome only rarely [81, 82], leading to the hypothesis that N-terminal APC truncations enhance tumorigenicity in a dominant-negative manner. To test the requirement of truncated APC for tumor formation, Cheung *et al.* made a mouse model with complete deletion of all 15 *Apc* exons ($Apc^{\Delta e1\text{-}15}$) [83]. $Apc^{\Delta e1\text{-}15/+}$ mice develop intestinal polyps of the same distribution and morphology as those seen in $Apc^{Min/+}$ mice, but with increased frequency. Polyps from $Apc^{\Delta e1\text{-}15/+}$ mice had lower levels of Apc^{+} mRNA compared to normal tissue, consistent with a requirement for loss of the wild-type allele for intestinal tumor development. $Apc^{\Delta e1\text{-}15/+}$ mice also develop more severe anemia than do $Apc^{Min/+}$ mice, and one $Apc^{\Delta e1\text{-}15/+}$ mouse developed a mammary tumor. Female $Apc^{\Delta e1\text{-}15/+}$ mice showed more severe phenotypes than did males. Polyps from $Apc^{\Delta e1\text{-}15/+}$ mice had lower mRNA levels of Wnt target genes *Axin2* and *c-Jun*, and *β-catenin* than did polyps from $Apc^{Min/+}$ mice [83]. Although puzzling in terms of the underlying mechanism, this observation is consistent with the hypothesis that there is a level of Wnt signaling optimal for polyp formation, and Wnt signling in excess of this level inhibits polyposis [83].

Apc mouse models with interstitial Apc mutations

Two mouse models have been recently described in which the engineered mutations result in changes within, rather than truncation of, Apc protein: $Apc^{mNLS/mNLS}$ and $Apc^{\Delta SAMP}$ mouse models.

$Apc^{mNLS/mNLS}$ model

Apc is perhaps best known as a Wnt signal antagonist. In this capacity, Apc is a component of a cytoplasmic complex that targets the oncoprotein β-catenin for proteasomal degradation [38]. APC also shuttles between the nucleus and the cytoplasm, aided by at least 2 nuclear localization signals (NLS) and 5 nuclear export signals (NES) [84]. Studies using cultured cells indicate that APC and β-catenin can interact in the nucleus, resulting in transcriptional repression of Wnt target genes and inhibition of cellular proliferation [85, 86]. In addition, nuclear APC interacts with Topoisomerase IIα, a critical enzyme required for DNA replication and a target for traditional cancer chemotherapeutics [87]. Moreover, APC has a role in DNA repair and synthesis [88, 89]. To study the role of nuclear APC in tissue homeostasis and tumor suppression, a mouse model was generated in which nuclear import of Apc is compromised via the introduction of mutations into both NLSs (Apc^{mNLS}) [90]. $Apc^{mNLS/mNLS}$ mice are viable, with no alterations in lifespan. Compared to $Apc^{+/+}$ mice, intestinal epithelia from $Apc^{mNLS/mNLS}$ mice were more proliferative and showed higher levels of Wnt target gene mRNA. In addition, $Apc^{Min/+}$ mice develop more and larger intestinal tumors when they also harbor the *Apc*^{mNLS} allele ($Apc^{mNLS/Min}$). Together, studies using the Apc^{mNLS} model support a role for nuclear Apc in inhibition of proliferation, Wnt signaling, and tumorigenesis [90].

$Apc^{\Delta SAMP}$ model

To directly examine the contribution of the Apc C-terminus to tumor suppression in the $Apc^{1322T/+}$ mouse model, the Tomlinson lab generated a mouse that expresses Apc lacking the amino acids 1322 to 2005 ($Apc^{\Delta SAMP}$) [91]. This Apc deletion eliminates all but the first 20-a.a. repeat and all SAMP motifs, but retains the C-terminal region of Apc. Phenotypes of the $Apc^{1322T/+}$ and $Apc^{\Delta SAMP}$ mice were identical with regard to polyp

number, distribution, size, and morphology, severity of dysplasia, differentiated and stem cell populations, and expression of Wnt target genes. These authors concluded that the C-terminal region of Apc is not involved in the suppression of intestinal adenoma in mice [91].

Changing the level of Apc expression

The Taketo laboratory generated two Apc mouse models with reduced *Apc* expression by inserting a neomycin cassette into *Apc* intron 13 in either reverse orientation (Apc^{NeoR}) or forward orientation (Apc^{NeoF}) [92, 93]. The neomycin cassette disrupts an enhancer and reduces the level of full-length Apc expressed from the mutant allele to 20% of normal levels for Apc^{NeoR} , and to 10% for Apc^{NeoF}. Both alleles are embryonically lethal in the homozygous state. By the age of 15 months, $Apc^{NeoR/+}$ and $Apc^{NeoF/+}$ develop intestinal polyps with relatively low incidence (19% and 50%, respectively) and multiplicity (0.26±5.4 and 1.09±8.5 polyps per mouse, respectively). The polyps in Apc^{NeoR} and Apc^{NeoF} mice display loss of the wild-type *Apc* allele and have less β-catenin stability and accumulation of nuclear β-catenin than do polyps from $Apc^{\Delta 716/+}$ mice [92, 93].

A transgenic mouse expressing truncated Apc

Based in part on the tendency of *APC* mutations in severe forms of FAP syndrome to result in truncation of the C-terminal half of Apc, and the ability of the truncated form of APC to bind to the full-length allele, it was proposed that particular APC truncations act in a dominant-negative manner [94]. A direct test of this hypothesis revealed no increased polyp susceptibility in mice carrying a transgene encoding Apc amino acids 1-716, even though the truncated Apc protein was detected in intestinal cells.

It is possible that dominant -negative Apc truncations lead to increased CIN and enhanced loss of the wild-type allele. Since mice in this experiment had two wild-type *Apc* alleles, loss of one would still leave one functional *Apc* allele and thus no increase in polyp formation. To explore this possibility, the transgene for truncated Apc was introduced into $Apc^{\Delta 716/+}$ mice [73]. Because intestinal tumor number, distribution, and morphology were the same in $Apc^{\Delta 716/+}$ mice with and without the truncated Apc transgene, it was concluded that truncated Apc does not act in a dominant-negative manner [73].

Conditional Apc mouse models

Mouse models with germline *Apc* mutations have been useful to probe many aspects of Apc biology, especially in intestinal tumorigenesis. However, most of these models are limited by a short life span, the predominance of intestinal phenotypes, and embryonic lethality in the homozygous state. To study functions of Apc at different developmental stages and in organs other than the intestine, investigators have developed mice with conditional *Apc* mutations [95]. A critical component of most conditional systems is Cre recombinase, which induces recombination between two *lox*P1 sites, to cause excision of the DNA between these sites. In conditional *Apc* mouse models, *lox*P1 sequences are inserted into introns of the mouse *Apc* gene flanking particular exon(s). In the presence of *Cre,* excision of the *lox*-flanked DNA leads to a frameshift mutation and truncation of the Apc protein. The specificity of this *Apc* mutation is achieved by placing Cre under control of a tissue- or developmental stage-specific promoter or an inducible promoter, or by infecting tissues with Cre-expressing Adenovirus [96]. Table 2.2 summarizes different conditional Apc mouse models.

Table 2.2: Mouse models with conditional Apc mutations

Mouse	Apc mutation	Organ	Developmental stage	Notes	Ref
Apc^{580S}	Floxed exon 14 results in stop codon at a.a. 580	Colon and rectum	Adult	Cre is delivered via Adenovirus vector injected in the colon through the anus. Develop colon adenomas in the distal 3 cm of the colon. Malignant transformation is seen in old lesions.	[77]
AhCre-Apc$^{fl/fl}$	Floxed exon 14 results in stop codon at a.a. 580	Small intestine, large intestine. Possibly the liver	Adult	Cre is expressed under Cyp1A promoter when mice were injected with β-naphthoflavone. Upregulation in Wnt signaling. Intestinal cell differentiation, proliferation, migration, and apoptosis were disrupted. Mice died after 4 days after induction.	[97]
Math1-Cre-Apc$^{Fl/Fl}$	Excision of the last exon	Cerebellum	Day 12.5 embryonic	Cre is expressed under Math-1 promoter in Granule cells in the cerebellum. No tumor, cerebellar cortical hypoplasia, impaired motor coordinator and ataxia	[98]
MMTV-Cre-Apc$^{flox/flox}$ *Pten*$^{flox/flox}$	Floxed exon 14 results in stop codon at a.a. 580	Salivary glands	??	Cre is expressed under MMTV promoter on B6X129 background. In this background, MMTV promoter is active in salivary gland and less active in mammary gland.	[99]

				Salivary gland tumors only with *Pten* deletion.	
LckCre-Apc$^{lox/lox468}$	Excision of exons 11 &12 resulting in frameshift splicing exons 10-13 and truncated Apc (468 a.a.)	Thymus	Starts at CD44-CD25+ double-negative 3 (DN3) stage and complete by DN4 stage of lymphocyte development	Cre is expressed under Lck promoter during the development of thymocytes. Thymic atrophy, reduced T-lymphocyte receptor rearrangement, increasing proliferation of pre-T cells, chromosomal segregation defects, T-cell developmental delay.	[100]
K14-Cre-Apc$^{CKO/CKO}$ *K14-Cre-Apc$^{CKO/+}$*	Floxed exon 14 results in stop codon at a.a. 580	Ectodermal derived tissues including mammary glands	Day 9.5 embryonic	Cre is expressed under Keratin-14 promoter in epidermal tissues. Growth retardation, premature death, abnormalities in epidermal derived tissues including: hair follicles, cornea, and teeth. Thymus hypoplasia, squamous metaplasia in the thymus (homozygous), mammary tumors in 76.5% in heterozygous females.	[76, 101]
Ahmr2-Cre-Apc$^{flox/flox}$	Floxed exon 14 results in stop codon at 580	Uterine stroma (in females) Sertoli cells (in males)	Fetus	Cre is expressed under anti-Mullerian hormone type II receptor in mesenchyme of fetal Mullerian duct. Progressive uterine hyperplasia and endometrial carcinoma. Apc has a	[27, 102]

				cell-non-autonomous role as an endometrial tumor suppressor protein. Large spleens (in females); abnormal spermatogenesis, loss of the apical part of Sertoli cells, disruption of tight junctions, no tumors (in males).	
Apc$^{\Delta ex14/\Delta ex14}$	Floxed exon 14 results in stop codon at a.a. 580	Liver	Adult	Cre under CMV promoter is delivered using Adenovirus injected intravenously. High viral dose causes hepatomegaly, hepatocellular hyperplasia and death. Low viral dose causes hepatocellular carcinoma	[103]
Vil-CreER^{T2}-*Apc*$^{lox/lox}$	Floxed exon 14 results in stop codon at a.a. 580	Small and large intestine	Adult	Cre is expressed under Villin promoter when the mice are injected with Tamoxifen. Upregulation of Wnt signaling, increased proliferation and apoptosis, decreased migration, increased number of cells committed to Paneth cell differentiation.	[104]
AhCre-Apc$^{fl/fl}$	Floxed exon 14 results in stop codon at a.a. 580	Kidney	Day 14.5-18.5 embryonic	Cre is expressed under Cyp1A promoter with no β-naphthoflavone induction. Renal carcinoma in ~1/4 of mice at 6 months,	[105]

				increased incidence with co-existence of p53 mutations	
WAP-Cre-Apc$^{CKO/CKO}$ *WAP-Cre-Apc$^{CKO/+}$*	Floxed exon 14 results in stop codon at a.a. 580	Lactating epithelial cells	Lactation	Cre is expressed under WAP promoter (Whey Acidic Protein). Mammary tumors in nulliparous and multiparous females (less than20%)	[76]
Col2a1-Cre-Apc$^{15lox/15lox}$	Excision of the last exon	Mesenchymal cells	Day 9.5 embryonic in sclerotome Day 12.5- 16.5 embryonic in chondrogenic and osteogenic cells.	Cre is expressed under Col2a1 (collagen-2a-1) in mesenchymal cells. Embryonic lethal, defective cartilage and bone differentiation	[1061]
Apc$^{CKO/CKO}$-LSL-Kras	Floxed exon 14 results in stop codon at a.a. 580	Distal colon	Adult	Cre is delivered via adenovirus vector injected in the colon through the anus, resulting in excision of Apc exon 14 and expression of mutant constitutively active Kras. Adenocarcinoma in the distal colon that show spontaneous metastasis to the liver after 24 weeks	[107]
Ahmr2-Cre-Apc$^{15flox/15flox}$	Excision of the last exon	Uterine myometrium	??	Cre is expressed under anti-Mullerian hormone type II receptor in mesenchyme of fetal Mullerian duct. Myometrial defects, dystocia, reduced number of endometrial glands	[1081]
Pgr-Cre-Apc$^{flox/flox}$	Excision of the last exon	Uterine endometrium&	??	Cre is expressed under progesterone receptor. Myometrial	[1081]

		myometrium		and endometrial defects, endometriosis interna-like changes	
FabplCre; Apc$^{15lox/+}$	Excision of the last exon	Distal small intestine and large intestine	??	Cre is expressed under fatty-acid binding protein-1 (Fabp1) promoter in some cells. Develop adenoma and adenocarcinoma mainly in large intestine	[79]
Pms^{2}-*Apc*$^{CKO/+}$	Floxed exon 14 results in stop codon at a.a. 580		??	Out-of-frame Cre that reverts back to frame stochastically. Rate of transformation is higher in Apc$^{1638N/CKO}$ and Apc$^{Min/CKO}$ mice relative to Apc$^{CKO/+}$ mice	[109]

References marked by the same color refer to studies done using the same *Apc* conditional mutation

What have we learned and what remains to be learned using Apc mouse models

The Apc mouse models have been valuable tools for studying the role of Apc in intestinal homeostasis and tumor suppression. They have aided in discovery of various pathways important in colon carcinogenesis. Apc mouse models were also useful for testing the effect of various environmental and genetic factors on intestinal tumorigenesis, and for testing potential chemoprevention and therapeutic agents. The many positive contributions of Apc mouse models have been reviewed elsewhere [80, 110]. As with most experimental systems, deeper investigation of Apc mouse models has led to increased understanding, but has also revealed more questions to be answered. Some of these mysteries and challenges for future investigation will be highlighted here.

What variables control polyp distribution in intestines of different Apc mouse models?

Tumors in the first Apc mouse model, $Apc^{Min/+}$, occur mainly in the small intestine, while germline mutation of *APC* in FAP patients results in tumors predominantly in the large intestine. The Apc rat model (PIRC) has tumors in both small and large intestines [5, 57, 111]. In addition to this inter-species variation, mouse models with different germline Apc mutations show different distributions of intestinal polyps.

Analysis of $Apc^{Min/+}$ mice with different genetic backgrounds has led to the explanation that polyp distribution is somehow linked to the mechanism by which the wild-type *Apc* allele is lost [54]. Haigis *et al.* showed that in a B6 background, $Apc^{Min/+}$ mice develop polyps mainly in the ileum, and loss of the wild-type *Apc* allele occurs by means of LOH. In an AKR background, $Apc^{Min/+}$ mice develop polyps predominantly at the ileo-cecal junction, and inactivation of the wild-type *Apc* allele is achieved through

allelic silencing. In the B6 background, $Apc^{Min/+}$ mice with additional mutations that inactivate the mismatch repair gene *Mlh* develop polyps all over the small intestine, and loss of the wild-type *Apc* allele is achieved through a point mutation. $Apc^{1638N/+}$ mice develop polyps in a similar distribution, and appear to retain the wild-type *Apc* allele [54].

Mechanistically, two models that are not mutually exclusive could account for the correlation between polyp distribution and loss of the wild-type *Apc* allele. In the first model, the molecular machinery in different intestinal regions determines the mechanism of the second *Apc* "hit" and hence the distribution of polyps. This model is supported by the finding that polyps in which the wild-type *Apc* allele is inactivated by the same mechanism (eg. $Apc^{Min/+}$ $Mlh^{-/-}$ and $Apc^{1638N/+}$ mice) have the same distribution [54]. However, the finding that both $Apc^{1322T/+}$ and $Apc^{Min/+}$ mice lose the wild-type *Apc* allele through LOH, yet have different polyp distributions, does not support this model. A second model proposes that polyp growth is dictated by the *Apc* status but also by the particular environment of the different intestinal regions, independent of the mechanism of the second *Apc* mutation. Supporting this hypothesis, $Apc^{\Delta 716/+}$ mice with an additional mutation of Cdx2 exhibit more colonic and fewer small intestinal polyps, yet whether or not Cdx2 is mutated, loss of the wild-type *Apc* allele occurs via LOH [112]. Similarly, a colonic shift of polyps has been described in $Apc^{Min/+}$ mice with an additional *BubR1* mutation, although the mechanism of loss of the wild-type Apc allele in these mice was not reported [113]. Mutation of both *Cdx2* and *BubR1* increases chromosomal instability and changes the proliferation and apoptotic indices in intestines of $Apc^{\Delta 714/+}$ and $Apc^{Min/+}$ mice, respectively [112, 113]. Further support for the second model comes from $Apc^{Min/+}$

mice in a 129/Sv background, where additional mutations that inactivate Smad3 result in more colonic tumors than in $Apc^{Min/+}$ mice; yet in both cases, loss of the wild-type *Apc* allele is achieved through LOH [114]. Finally, PPARγ agonists increase colonic but not small intestinal tumors in $Apc^{Min/+}$ mice [30, 31]. PPARγ is expressed in higher quantities in the colon and cecum relative to the small intestine, which might account for this differential effect [31].

Perhaps some of these mechanisms can be clarified by studying $Apc^{Min\text{-}FCCC}$ mice that were generated by mating C57Bl/6J $Apc^{Min/+}$ mice with C57Bl/6JNIcr mice. $Apc^{Min\text{-}FCCC/+}$ mice develop more colon polyps than do $Apc^{Min/+}$ mice, but the molecular basis behind this polyp shift has not been determined [115]. Further clarification of the underlying mechanism that controls polyp distribution might be achieved through careful analysis of $Apc^{\Delta 14/+}$ and $Apc^{580D/+}$ mice, which carry the same mutation but appear to have different polyp distributions. $Apc^{\Delta 14/+}$ mice develop more colonic polyps than do $Apc^{Min/+}$ mice, and $Apc^{580D/+}$ mice develop a similar number of colonic polyps as do $Apc^{Min/+}$ mice [76, 78]. An important caveat to consider is that there are no published studies that directly compare $Apc^{580D/+}$ and either $Apc^{\Delta 14/+}$ or $Apc^{Min/+}$ mice.

Why do different Apc mutations result in altered multiplicity of intestinal polyps?

There is evidence that variation in intestinal polyp distribution among different mouse models can be explained by the nature of the *Apc* mutations in theses polyps. We propose that differences in polyp number in the various mouse models results from one or more of the following contributing factors:

1- ***Different rates and mechanisms of wild-type Apc allele loss (e.g. LOH, mutations of the wild-type copy of Apc, gene silencing).***

As discussed above, the mechanism of loss of the second wild-type *Apc* allele is different in different Apc mouse models. Because this second *Apc* "hit" is thought to be required for polyp initiation [11, 53, 75], the rate at which second hits occur will directly affect the number of intestinal polyps. Increasing the expected rate of these second "hits" through introduction of genomic instability, X-ray exposure, or injection with a mutagen, significantly increases the number of polyps in $Apc^{Min/+}$ and Apc^{1638N} mice [16, 47, 52, 116, 117]. Mutations in *Apc* might also induce CIN, which would theoretically affect the rate of the wild-type *Apc* allele loss [44].

In $Apc^{1638N/+}$ mice, which develop relatively few intestinal polyps, the second *Apc* "hit" is usually an inactivation of the wild-type *Apc* allele, which is predicted to be a rare event [54]. On the other hand, $Apc^{Min/+}$ mice, where the wild-type *Apc* allele is lost by means of a more frequent LOH event, develop considerably more polyps [54]. Loss of the wild-type *Apc* allele in both $Apc^{Min/+}$ and $Apc^{1322T/+}$ mice, however, is reported to occur via LOH, yet these two mouse models have widely different polyp numbers [69]. Furthermore, several groups have reported that although loss of both *Apc* alleles is required to activate Wnt signaling, this *Apc* loss is not sufficient for full activation of Wnt signaling (as assessed through nuclear translocation of β-catenin) [118-120]. Although the mechanism and rate by which the wild-type *Apc* allele is lost might contribute to the control of intestinal polyp number in Apc mouse models, it is unlikely that these are sole defining parameters.

2- Different rates of polyp growth due to differences in Wnt signaling

Polyps must reach a certain size to be detectable. If two polyps are initiated at the same time, the more rapidly growing polyp will be detectable earlier than will be the

slowly growing polyp. The most recognized function of Apc is to antagonize the Wnt signaling pathway through inhibition of β-catenin's activity as a transcription co-factor [38]. As Wnt signaling drives cellular proliferation, we might expect that mice with different *Apc* mutations, and therefore different levels of Wnt signal activation, would display corresponding changes in cellular proliferation. This simplistic interpretation is challenged by findings in the Apc mouse models. First, the correlation between Wnt signaling activity and cellular proliferation does not appear to be linear. On one hand, the frequency of polyps from $Apc^{\Delta 716/+}$ mice, which have highly elevated Wnt signaling, is higher relative that of $Apc^{Min/+}$ mice with less Wnt signal elevation [74]. Polyps from $Apc^{NeoR/+}$ and $Apc^{NeoF/+}$ mice, which have less β-catenin stability and accumulation of nuclear β-catenin, are also less frequent than are polyps of $Apc^{\Delta 716/+}$ mice with more Wnt signal elevation [92, 93]. However, $Apc^{1322T/+}$ and $Apc^{\Delta e1\text{-}15/+}$ mice have more polyps than do $Apc^{Min/+}$ mice, yet the level of Wnt signaling is higher in Apc^{Min} polyps, consistent with the "just right hypothesis" [70, 83]. Second, the correlation between mutations in *Apc* and Wnt signaling activity is not fully understood. For example, complete deletion of *Apc* in polyps results in less Wnt signaling activity than in polyps genotyped as $Apc^{Min/Min}$ [83]. Further, Wnt signal up-regulation in $Apc^{mNLS/mNLS}$ and $Apc^{1638T/+}$ mice does not result in robust polyp development [60, 90]. Direct comparison of Wnt signaling activities and proliferation rates in $Apc^{neoF/+}$, $Apc^{neoR/+}$, $Apc^{mNLS,mNLS}$, $Apc^{1638T/+}$, $Apc^{1322T/+}$, and $Apc^{\Delta e1\text{-}15/+}$ mice would help to establish the contribution of Wnt signaling and polyp growth to phenotypic variation. Finally, $Apc^{\Delta 14}$ and $Apc^{\Delta 242/+}$ mice were reported to exhibit more severe intestinal polyposis than do $Apc^{Min/+}$ mice [71, 72, 78]. $Apc^{\Delta 14}$ and $Apc^{\Delta 242/+}$ mice have mutations 5' to that of $Apc^{Min/+}$ mice, and Apc should

therefore lack portions of the armadillo repeats. A direct assessment of Wnt signaling between $Apc^{\Delta 14}$, $Apc^{\Delta 474/+}$, and $Apc^{\Delta 242/+}$ mouse models would be informative.

3- Different abilities to evade growth inhibitory effects

Another explanation of variation in polyp number among different Apc mouse models is negative selection of particular *Apc* genotypes. This negative selection could contribute to the "Just right" hypothesis. Support for negative selection contributing to polyp phenotypes is provided by the observation that addition of *Cdx2* or *BubR1* mutations to $Apc^{\Delta 716/+}$ or $Apc^{Min/+}$ mice, respectively, results in reduced polyp multiplicity and increased apoptotic indices in the small intestines, despite the increased proliferation index in these cells [112, 113].

4- Distinctive effects on differentiation

Another possible source of differences in intestinal polyp number in different Apc mouse models is the effect of *Apc* genotypes on enterocyte differentiation. For instance, compared to $Apc^{Min/+}$ mice, $Apc^{1322T/+}$ mice have a higher proportion of Paneth cells and cells that express stem cells markers (Lrg5, Bmi1, Msi1 and CD44), not only in adenomas (presumably the result of LOH) but also in apparently normal intestinal epithelial cells [70]. It is possible that different cell fates resulting from different Apc genotypes alter tumor initiation or growth. Again, Wnt signaling is one of several factors proposed to affect differentiation.

5- Contributions of other genetic or environmental factors with potentially differential effects dependent on genotype

The effect of genetic and environmental factors on intestinal polyp multiplicity in Apc mouse models, particularly in $Apc^{Min/+}$ mice, has been well established. Polyp

multiplicity in $Apc^{Min/+}$ mice varies greatly (20-100/mouse) across laboratories [7, 35]. This inconsistency might result from variations in diet, emergence of genetic modifiers, and even from different methods of polyp detection. The many genetic modifiers described in $Apc^{Min/+}$ mice potentially have different effects, dependent on the particular *Apc* allele, although this has not been examined thoroughly. In addition, modifier genes are present and can even emerge in what is considered a congenic strain [121]. Environmental factors, such as intestinal flora, might also contribute to phenotypic variation [8]. While intestinal flora appear to slightly increase the number of polyps in $Apc^{Min/+}$ mice [122], $Apc^{\Delta 14/+}$ mice raised in pathogen-free conditions showed significant increases in intestinal polyp number. It is important to note that mice with *Apc* mutations might respond differently to intestinal flora than do wild-type mice [123]. Another major environmental factor that clearly affects the mouse phenotype is diet. The effect of diet on the number of polyps in $Apc^{Min/+}$ mice has been shown in several different studies [124-126]. Although typically defined, the concentration of various vitamins, fiber, and total fat varies greatly between laboratory mouse diets. In our own experience, switching the mouse diet from Lab diet 5001 (Purina) to Harlan 2018 had a dramatic effect on polyp multiplicity in our $Apc^{Min/+}$ mouse colony. Notably, the new diet has increased fat by 24% and decreased fiber, vitamin D, and folic acid by 42%, 67%, and 44%, respectively. We compared the number of polyps in 10 $Apc^{Min/+}$ mice fed the new diet to the recorded number of polyps in 25 age-matched mice on the old diet. We found that the polyp burden per mouse significantly increased from 45.9±4.5 in $Apc^{Min/+}$ mice on the old diet to 81±9.3 in $Apc^{Min/+}$ mice on the new diet (p= 0.0006). As a second dramatic example from personal experience, we obtained two male $Apc^{1322T/+}$ mice from the

Tomlinson laboratory at age 5 weeks. At the age of 16 weeks, the number of polyps in one mouse was only 50% of the number reported by the Tomlinson group [69]. By the second generation of breeding with our C57BL/6J mice, the $Apc^{1322T/+}$ mice had roughly one-third the number of polyps originally reported. This informal observation is reminiscent of the epidemiological study that showed that the incidence of colon cancer in U.S. immigrants moved toward that of U.S. natives, with second-generation immigrants trending even closer to that of US natives [127]. Diet likely plays a major factor in the phenotypic variation from one laboratory to another. Unfortunately, these inter-laboratory variables confound direct comparison of the phenotypes of different mouse models studied in different laboratories.

6- Differences in cellular migration and adhesion

Apc interaction with cytoskeletal components, including actin filaments and microtubules, is thought to affect cell adhesion and migration [41, 128]. It was predicted that decreased cellular adhesion and migration in cells with *APC* mutations would enhance tumor formation [129]. Apc interacts with cytoskeletal protein through its C-terminal domain, which is absent in most Apc mouse models (figure 2.1). Adding the C-terminal Apc domain to Apc^{1322T} (as in $Apc^{\Delta SAMP}$ mice) does not change the phenotype [91]. However, it has been proposed that effects on the cytoskeleton alter tumor progression at later stages (e.g. invasion and metastasis) [130], which don't occur in most Apc mouse models. Current evidence in support of a direct role of the C-terminal region of Apc in observed intestinal phenotype variation among different Apc mouse models is lacking.

7-Differences in technologies used to generate the mouse model

Additional contributing factors for consideration include the different technologies used to generate these various Apc mouse models. The $Apc^{Min/+}$ mouse was generated by chemical mutagenesis that resulted in a single base-pair change in the *Apc* gene [4]. Many other models, such as Apc^{1309}, Apc^{1638N} and Apc^{1638T}, were generated through insertion of an antibiotic-resistance gene into the *Apc* gene, thus introducing a nonsense mutation [46, 60, 68]. In Apc^{neoF} and Apc^{neoR} alleles, the antibiotic-resistance gene disrupts an enhancer sequence in an intron 13 [92, 93]. The Apc^{1322T} model was generated via Cre-Lox-mediated deletion, and the Apc^{mNLS} model contains mutations "knock-ins" to the *Apc* gene, with the antibiotic-resistance gene subsequently removed by means of Cre-Lox-mediated deletion [69]. The $Apc^{1638N/+}$ and $Apc^{1638T/+}$ models, which differ only by orientation of the inserted neomycin-resistance gene, provide clear evidence for the contribution of extraneous DNA to phenotypic variation [60]. $Apc^{1638N/+}$ mice express so little truncated Apc protein that they might be considered virtually null [53]; yet the described phenotype of $Apc^{1638N/+}$ mice is not similar to that of the $Apc^{\Delta e1-15}$ model, which has a complete deletion of the *Apc* gene [53, 83]. The neomycin-resistance gene clearly affects the phenotypes of these mice and if inserted in reverse orientation, might affect not only Apc expression, but also expression of genes upstream of *Apc*. Another example of a possible effect of the induced mutation is seen in $Apc^{\Delta 474/+}$ mice, which have a duplication of *Apc* exons 7-10. This feature makes it difficult to dissect the effects of duplication of four *Apc* exons from the effects of deleting the rest of the protein [72].

8-Differences in expression of the mutant allele

When analyzing the phenotypes of different Apc mouse models, another consideration is the level of expression of the mutant allele. Although normal expression levels of truncated Apc protein have been verified in $Apc^{\Delta 716}$, $Apc^{Min/+}$, Apc^{1322T}, and Apc^{1638T} mice, this is not universally the case [60, 69, 131]. In Apc^{580D}, $Apc^{\Delta 14}$, $Apc^{\Delta 474}$, and $Apc^{\Delta 242}$ models, the truncating mutation occurs before the final exon (15), and thus there is the possibility of nonsense-mediated RNA decay. Remarkably, the truncated Apc was not detected in intestinal polyps from $Apc^{\Delta 580/+}$ mice and ES cells from $Apc^{\Delta 15/+}$ mice [76, 79], which suggests that these might also be virtually null alleles. A related consideration is the effect of the introduced mutation (and possibly the antibiotic selection cassette) on Apc folding. Although most of the Apc protein is thought to be natively unfolded [132], the effects of mutations on inherently folded domains of Apc and the consequences of potential folding defects in relation to phenotype, are not understood.

Why do different Apc mutations sometimes cause extra-intestinal phenotypes?

As with the intestinal phenotype, the underlying mechanism for variation in extra-intestinal phenotypes in different Apc mouse models is not completely understood. Some phenotypes, such as anemia, seem to correlate with the severity of intestinal polyposis. In contrast, mammary gland tumors in Apc mouse models appear to correlate with the severity of polyposis in only a few cases, such as in the $Apc^{Min/+}$ and $Apc^{\Delta 474/+}$ models. Very few $Apc^{Min/+}$ mice develop mammary tumors, whereas $Apc^{\Delta 474/+}$ mice develop mammary tumors at a rate that is almost double that seen in $Apc^{Min/+}$ mice [6, 72]. $Apc^{mNLS/Min}$ females also have enhanced mammary tumorigenicity (see chapter 5). There are no reports of mammary tumor development in Apc mouse models with the most

severe intestinal polyposis ($Apc^{\Delta 714}$, Apc^{1322T}, and $Apc^{\Delta SAMP}$), however [69, 74, 91]. Perhaps mice with severe polyposis die relatively early, before mammary tumors have a chance to develop. $Apc^{1572T/+}$ mice, which develop no intestinal polyps, have a fully-penetrant mammary tumor phenotype in females. There is a hypothesis that Wnt signaling must be "just right" to support mammary tumorigenesis [62]. Evidence supporting this hypothesis comes from $Apc^{\Delta 580/+}$ and K14-cre-$Apc^{CKO/+}$ mice, where mutations in the wild-type *Apc* allele in mammary tumors cluster around codon 1530 (eliminating all 3 SAMP repeats from the truncated Apc) [101]. K14-cre-$Apc^{CKO/+}$ mice are a conditional model in which the $Apc^{\Delta 580}$ allele is expressed only in ectodermal derived tissues including the mammary gland [76, 101]

Apc rat models

To overcome some of the limitations of Apc mouse models, Amos-Landgraf *et al.* used an ENU mutagenesis screen to generate a rat model with a germline nonsense mutation at Apc codon 1137 (Apc^{am1137}) [111]. Rats homozygous for the Apc^{am1137} allele die as embryos. $Apc^{am1137/+}$ rats develop both small intestinal and colonic polyps with 100% penetrance, and are called "PIRC" rats for Polyposis In Rat Colons [111, 133]. The polyps in PIRC rats are adenomas with malignant changes, with local invasion seen in old rats. No signs of metastasis have been detected in these rats. As seen in humans with germline *Apc* mutations, the polyps from PIRC rats show β-catenin nuclear translocation in advanced but not in early adenomas. As with $Apc^{Min/+}$ mice, intestinal polyps in PIRC rats show LOH. Because chromosome18, which carries the *Apc* gene in rats, is metacentric, pyrosequencing could be used to demonstrate that LOH in PIRC rats predominantly occurs by means of homologous recombination [111]. The greater width

of rat intestines and colons, relative to those of mice, allows for growth of larger intestinal tumors, which allows study of tumor progression beyond the early stage. Wider colons and higher colonic tumor multiplicities also facilitate longitudinal endoscopic studies of tumorigenesis [111]. Male PIRC rats have more polyps than do females [83]. Although most Apc mouse models do not show a gender bias, male $Apc^{Min\text{-}FCCC/+}$ mice also develop more colonic polyps than do females. In contrast, female $Apc^{\Delta e1\text{-}15/+}$ mice display more severe phenotypes than do males [83]. In humans, women appear to be slightly less affected by colon cancer than do men [134]. PIRC rats also show high incidence of jaw tumors, which are the main cause of morbidity in female PIRC rats [111]. This extra-intestinal phenotype has also been described in patients with FAP syndrome [135].

A second Apc rat model was generated by use of ENU as a chemical mutagen [136]. This rat model (Kyoto Apc Delta or KAD rat) has a nonsense mutation in the *Apc* gene, resulting in deletion of the C-terminal 321 amino acids. This terminal deletion does not appear to affect life expectancy even in homozygous KAD rats, and no spontaneous polyps develop in the intestines of these rats. However, KAD rats showed enhanced inflammatory-induced colon tumorigenicity, which suggests a Wnt-independent role of the C-terminal domain of Apc in tumor suppression [136].

In summary, rodent models with *Apc* mutations were first generated more than 2 decades ago, even before identification of human *APC*. Studies of 35 rodent models with germline and conditional *Apc* mutations has led to greater understanding of the role of Apc in development, differentiation, and homeostasis of intestinal epithelial cells. In addition, these models have allowed exploration of the role of Apc in intestinal and extra-

intestinal development and tumorigenesis. Mouse and rat models with germline Apc mutations have permitted experimental testing of different molecular pathways and investigation of genetic and environmental contributions to tumor formation, not only in the gastrointestinal tract but also in other tissues. These models have also facilitated testing different preventive and therapeutic agents in preclinical studies. However, additional work is required to clarify some of the less understood features of Apc rodent models so as to maximize their usefulness. Standardizing genetic and environmental variables and conducting comparative studies of these Apc models is expected to enhance our understanding of Apc and of cancer biology.

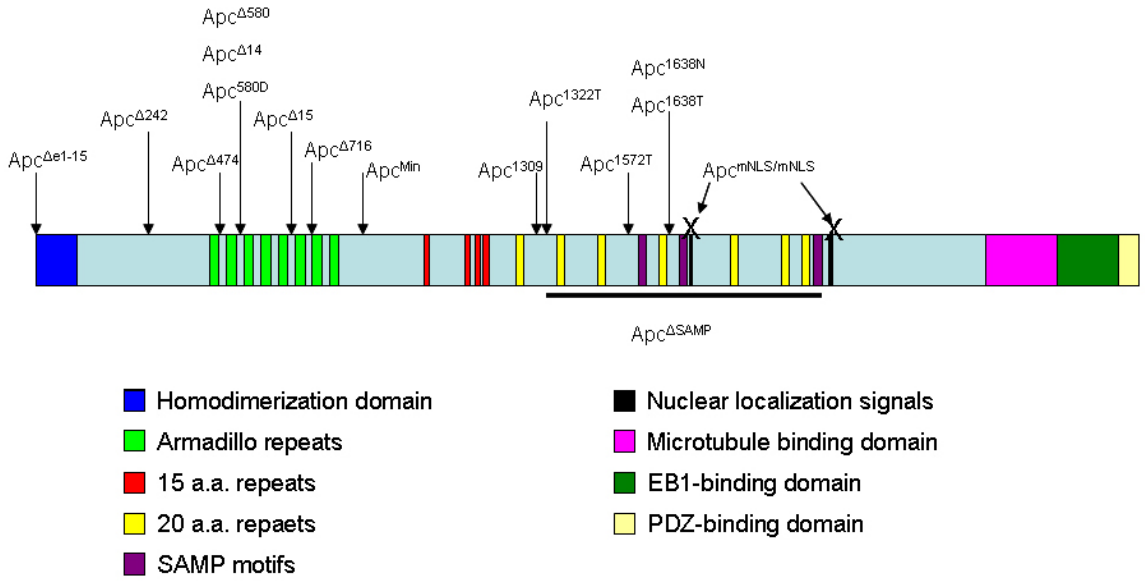

Figure 2.1: Apc protein structure and the location of germline mutations from various Apc-models

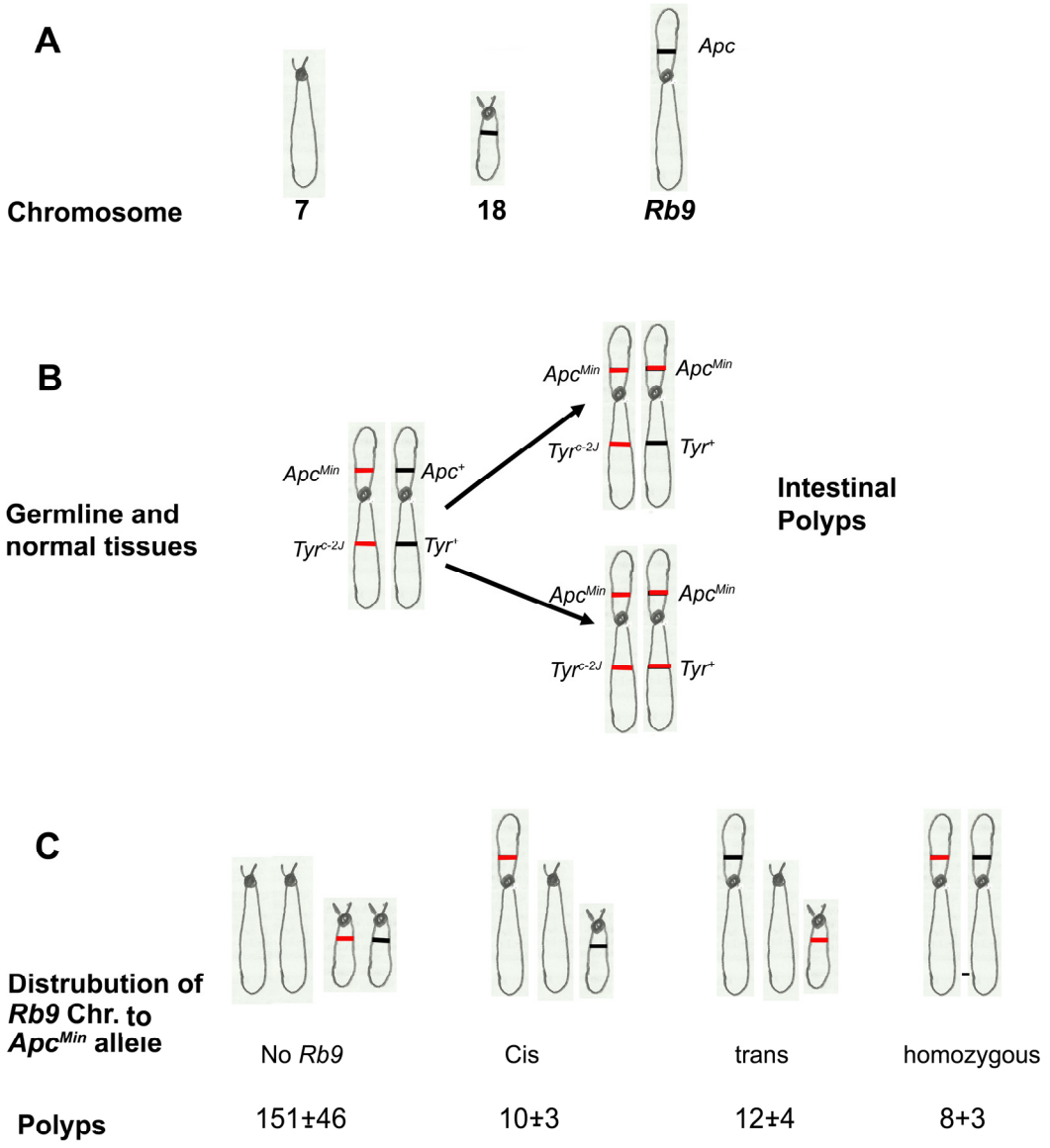

Figure 2.2: Using a Robertsonian translocation of chromosome 7 over 18 to test for the mechanism of LOH in Apc$^{Min/+}$ mice. (A) Schematic diagram for chromosomes 7, 18, and *Rb9*. The *Apc* locus on chromosome 18 is marked with a black line. **(B)** Schematic diagram of using a natural mutation on the chromosome 7 arm of the *Rb9* translocation chromosome to test if LOH in polyps from Apc$^{Min/+}$ is caused by somatic recombination or by mitotic non-disjunction. Left side shows the chromosomal arrangement and status of the *Tyr* and *Apc* alleles on both arms of the *Rb9* chromosome in both germline and normal tissues. On the right, the expected *Tyr* and *Apc* alleles on both arms of *Rb9* chromosome if LOH is the result of somatic recombination (upper result) or mitotic non-disjunction (lower). **(C)** Average polyp numbers in Apc$^{Min/+}$ mice is decreased when *Rb9* chromosome is present in cis, trans, or homozygous distribution with the *Apc^{Min}* allele. (Adapted from Haigis and Dove 2003 [13])

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Chapter 3

The data and opinions in this chapter were published previously and reformatted for this dissertation [1]

A knock-in mouse model reveals roles for nuclear Apc in cell proliferation, Wnt signal inhibition and tumor suppression

Abstract

Mutation of the tumor suppressor adenomatous polyposis coli (*APC*) is considered an initiating step in the genesis of the vast majority of colorectal cancers. APC inhibits the Wnt signaling pathway by targeting proto-oncogene β-catenin for destruction by cytoplasmic proteasomes. In the presence of a Wnt signal, or in the absence of functional APC, β-catenin can serve as a transcription co-factor for genes required for cell proliferation such as cyclin D1 and c-Myc. In cultured cells, APC shuttles between the nucleus and cytoplasm, with nuclear APC implicated in inhibition of Wnt target gene expression. Taking a genetic approach to evaluate the functions of nuclear APC in the context of a whole organism, we generated a mouse model with mutations that inactivate the nuclear localization signals of Apc (*Apc*^{mNLS}). Apc$^{mNLS/mNLS}$ mice are viable and fractionation of embryonic fibroblasts (MEFs) isolated from these mice revealed a significant reduction in nuclear Apc compared to Apc$^{+/+}$ MEFs. The levels of Apc and β-catenin protein were not significantly altered in small intestinal epithelia from Apc$^{mNLS/mNLS}$ mice. Compared to Apc$^{+/+}$ mice, Apc$^{mNLS/mNLS}$ mice displayed increased proliferation in epithelial cells from the jejunum, ileum, and colon. These same tissues from Apc$^{mNLS/mNLS}$ mice displayed more mRNA from three genes up-regulated in response to canonical Wnt signal, *c-Myc*, *Axin2*, and *Cyclin D1*, and less mRNA from *Hath 1* which is down-regulated in response to Wnt. These observations suggest a role for nuclear Apc in inhibition of canonical Wnt signaling and control of

epithelial proliferation in intestinal tissue. Furthermore, we found *Apc*$^{Min/+}$ mice, which harbor a mutation that truncates Apc, have increased polyp size and multiplicity if they also carry the *Apc*^{mNLS} allele. Taken together, this analysis of the novel Apc^{mNLS} mouse model supports a role for nuclear Apc in control of Wnt target genes, intestinal epithelial cell proliferation and polyp formation.

Introduction

The tumor suppressor protein adenomatous polyposis coli (APC) is large, with multiple subcellular localizations and functions. Mutation of the *APC* gene is considered the initiating event in the formation of most intestinal polyps, the precursors to colorectal cancer [2-4]. As such, an intense investigation of potential APC functions involved in tumor suppression has led to identification of APC as a Wnt signaling pathway antagonist [5]. In this capacity, APC is part of a cytoplasmic protein complex that targets the proto-oncoprotein β-catenin for proteasome-mediated destruction [6]. APC has also been observed in nuclei of cultured colon cells and colonic epithelia from human tissue using conventional and confocal immunofluorescent microscopy and immunoelectron microscopy [7-9]. Nuclear APC has three proposed roles in regulating Wnt signaling. First, nuclear APC binds to nuclear β-catenin and likely competes with transcription factor TCF/LEF for β-catenin binding [10]. Second, nuclear APC has been implicated in the nuclear export of β-catenin [11-13]. Finally, the interaction of nuclear APC with transcriptional corepressor CtBP further contributes to the modulation of Wnt signaling [14]. In addition to its role as a repressor of β-catenin, nuclear APC has been implicated in DNA synthesis and repair [15, 16].

Because APC is a large protein (~310 kDa), it is unable to simply diffuse into the nucleus but must instead be actively transported through nuclear pores [17, 18]. Several domains of APC have been proposed to mediate this nuclear entry, including two monopartite nuclear localization signals (NLS) in the central part of the protein [19], as well as an armadillo repeat region closer to the N-terminus [20]. In addition, phosphorylation of APC regulates its localization [19] and relative levels of nuclear and cytoplasmic APC appear to correlate with the proliferative status of a cell [21].

Although many mouse models have been generated with alterations in *Apc*, they either produce no Apc protein [22], full-length Apc at a reduced level [23], or a truncated Apc [24-30]. Elimination of the C-terminal portion of Apc leads to at least partial loss of function and mice homozygous for mutations that truncate Apc typically do not develop beyond embryonic day 8 [31]. To date, no mouse model with targeted disruption of the two Apc NLSs has been generated.

To assess the functions of nuclear Apc in the context of a whole organism, we introduced germline mutations into the mouse *Apc* gene that result in inactivation of the two characterized Apc NLSs (Apc^{mNLS}). Inactivation of the two APC NLSs was previously shown to attenuate the ability of full-length exogenous human APC to enter the nucleus [19]. In the present study, mice heterozygous ($Apc^{mNLS/+}$) or homozygous ($Apc^{mNLS/mNLS}$) for mutant Apc NLS were viable. Intestinal epithelial cells in $Apc^{mNLS/mNLS}$ mice showed increased levels of Wnt target gene expression and more proliferation compared to their wild-type littermates. For the past two decades, the $Apc^{Min/+}$ mouse has been a popular model organism to study *Apc* mutation-driven tumorigenesis [32]. $Apc^{Min/+}$ mice have a missense mutation in Apc that results in

truncation of the C-terminal 2/3 of the protein. $Apc^{Min/+}$ mice develop 20-60 intestinal polyps at the time of their natural death, ~120 days of age. We found that $Apc^{Min/mNLS}$ mice displayed significantly more intestinal polyps than $Apc^{Min/+}$ mice and these polyps were larger. Together, these results suggest that nuclear Apc participates in regulation of Wnt signaling, proliferation and tumor suppression.

Materials and methods:

Creating the Gene Replacement Vector

The homologous sequences of DNA used for the targeting construct were obtained from a lambda phage library of genomic DNA isolated from 129 mouse ES cells (generously provided by Kirk Thomas and Mario Capecchi, University of Utah). This library was screened for fragments of *Apc* containing the two primary *Apc* nuclear localization signals (NLSs). Following identification of an *Apc NLS*-containing plaque by hybridization to a radioactive probe, a 14 kb stretch of mouse genomic DNA (*Apc* exons 14, 15 and surrounding introns) was isolated from the phage and inserted into the pBluescript KSII+ vector (Stratagene). An *EcoRI* fragment containing both *Apc* NLSs was subcloned into the pUC19 vector. Mutations that inactivated each NLS and introduced novel restriction sites were inserted by PCR mutagenesis (Figure 3.1B). A second pUC19 vector was modified to destroy its *EcoRI* restriction site and introduce *Nhe I* and *Not I* sites. An 11,537 bp region of *Apc* (*NheI/NotI*) was subcloned into this modified vector. The *EcoRI* fragment of *Apc* containing wildtype NLSs was replaced by the same fragment with mutant NLSs. Using restriction sites for *KpnI* and *AflII* in the noncoding region 3' to *Apc* exon 15, a tACE-Cre-Neo^r cassette, flanked by two LoxP sites was introduced into the pUC19 vector containing the *Apc* gene fragment with

mutant *Apc* NLSs. This tACE-Cre-Neo^r cassette included a neomycin resistance gene (Neo^r) driven by the promoter for RNA polymerase II and was linked to a gene encoding Cre recombinase under control of the testes-specific murine angiotensin-converting enzyme (tACE) promoter [33]. The HSV thymidine kinase gene controlled by the phosphoglycerate kinase (PGK) promoter was inserted upstream of the *Apc* homology sequences in the pUC19 vector to create the 19,947 bp targeting vector (Figure 3.1A).

Electroporation into Embryonic Stem Cells

The targeting construct DNA was cleaved with Not I restriction endonuclease to linearize at a unique site 3' of the region homologous to *Apc*. Digestion with *Not I* was followed by two phenol/chloroform extractions before the linearized gene replacement vector was electroporated into R1 mouse embryonic stem cells [34] at the Transgenic and Gene-Targeting Institutional Facility at The University of Kansas Medical Center. Cells were selected for growth in 300μg/mL G418 (Cellgro) and 2μM ganciclovir (Roche). Following 8-10 days of culture in selective media, each surviving colony was picked and expanded into two separate wells. DNA was isolated from one well for genotype analysis and the cells in the remaining well were frozen in 96-well plates for injections.

Screening and Verifying Targeted ES cell lines

Identification of ES cell lines containing mutations in both *Apc* NLSs was accomplished using a two-step PCR screen and the following primer sets: wildtype *Apc* NLS1 forward and NLS2 reverse, wNLS1fw (5'-CTA AGA AAA AGA AGC CTA CTT CAC) and wNLS2rv (5'-GGC CTT TTC TTT TTT GGC ATG GC); mutant *Apc* NLS1 forward and NLS2 reverse, mNLS1fw (5'-GCA GCC GCG GCA CCT ACT) and mNLS2rv (5'-TTG AAG GCC TTT TTG CGG CC) (Figure 3.1C). To identify

homologous cassette insertion at the 3' end, a primer annealing to DNA in the LoxP/tACE promoter region of the tACE-Cre-Neo^{r} cassette (5'-CCT GGC CCA TGG AGA TCC AT) was used with a primer annealing to genomic *Apc* 3' of the targeting construct (5'-CAT ACC ACC CAC CAT CCC TA) or with a primer annealing to the 3' end of the targeting construct (TCT CCC ATT GCT TAT GGC AAC) as a positive control (Figure 3.1H). Cell lines were also screened for correct incorporation of the 5' end of the targeting construct using PCR. The reverse primers wNLS2rv and mNLS2rv were used in conjunction with the following primer that anneals to a region of genomic DNA upstream of the 5' end of the targeting construct: (5'-AAA TTG AAC TCA GGA CCT TCT C) (Figure 3.1F). PCR products digested with *SstII* produced a 6100bp DNA product if mutant *Apc* NLS1 was present (Figure 3.1G). Each cell line with correct homologous recombination was further evaluated by determining the karyotype of 40-50 cells.

Generation of Chimeric Mice

For the generation of chimeric mice, Apc$^{mNLS/+}$ ES cells were injected into C57BL/6J blastocysts at the Gene Targeting and Transgenic Facility at the University of Virginia. Male chimeric mice that carried the mutant copy of *Apc* were bred to C57BL/6J females originally obtained from The Jackson Labs (Bar Harbor, Maine). Black progeny were culled and agouti colored progeny were genotyped using tail DNA isolated following a protocol from The Jackson Laboratory (http://www.jax.org/imr/tail_nonorg.html) and PCR to screen for the presence of the mutant nuclear localization sites. Verification of selection cassette loss in the Apc$^{mNLS/+}$ progeny was conducted using PCR with the following primers: forward primer, 5'-TCG

GCC ATT GAA CAA GAT GGA-3' and reverse primer, 5'-ATT CGC CGC CAA GCT CTT CA-3' (Figure 3.1E).

Mouse Husbandry

Mice were maintained in the Animal Care Unit at the University of Kansas according to animal use statement number 137-01. The research complied with all relevant federal guidelines and institutional policies. The chimeric mice were bred with C57BL/6J mice from The Jackson Labs (Bar Harbor, Maine). $Apc^{mNLS/+}$ progeny from the original mating of male chimeric mice with female C57BL/6J mice were repeatedly backcrossed to the inbred C57BL/6J mice 10-18 times to generate a line that was considered to be congenic (N10-N18). Detailed records including the date of birth, lineage, coat color, sex, genotype and date of sacrifice were maintained for each individual mouse. For the survival curve, N1 male and female $Apc^{mNLS/+}$ mice were bred and F1 progeny were housed with same sex siblings for the duration of the study (n=18 for $Apc^{+/+}$; 19 for $Apc^{mNLS/+}$; and 18 for $Apc^{mNLS/mNLS}$ mice). Cause of death for mice in the survival study was not determined because mice were allowed to die naturally and thus most were not collected for many hours after their death. Mice were fed *ad libitum* with Purina Lab Diet 5001 and were housed in cages in adjoining animal rooms. Congenic mice weighed at different times between ages 6 and 14-weeks showed no significant differences comparing $Apc^{+/+}$ and $Apc^{mNLS/mNLS}$ mice. $Apc^{Min/+}$ mice were purchased from The Jackson Labs and were maintained by breeding males with C57BL/6J females. $Apc^{Min/mNLS}$ mice were generated by breeding male $Apc^{Min/+}$ mice (from Jackson Labs) with congenic (N10-N15) female $Apc^{mNLS/+}$ mice.

Mouse genotyping

After weaning, mouse pups were tagged with a metal ear tag or by injection of an implantable electronic transponder (Bio Medic Data Systems Inc.) into the subcutaneous space above the shoulders. The *Apc* genotype of each mouse pup was determined using isolated tail DNA and PCR to screen for the presence of the wild-type and mutant NLS coding sequences using the following primers: forward, 5'-TAGTGATGCGGTGAGTCCAA-3' and reverse, 5'-ACCAAGTCCAACAAGCATCC-3'. Reaction conditions were: 94°C for 5 min., 35 cycles of 94°C for 1 min., 54°C for 1 min., 68°C for 1 min., final 3 min. at 68°C. PCR products (295 bps) were cut with *Sac*II restriction enzyme. The restriction enzyme does not cut the wildtype allele but cuts the mutant allele into 2 fragments of 235 and 60-bp (Supplemental Fig. S 3.1). The Apc^{Min} allele was detected using the standard PCR protocol published by the Jackson Laboratories (http://jaxmice.jax.org).

Analysis of gross and microscopic pathology, polyp measurement

The gross and cellular histology of intestinal tissues were examined in the N1-N13 generations of Apc^{mNLS} mice and in fourteen-week old $Apc^{Min/+}$ mice and $Apc^{Min/mNLS}$ mice. For each mouse, GI tract from the stomach to the anal canal was dissected, opened longitudinally and fixed in 10% buffered formalin. Using a dissecting microscope, an investigator blind to the animal's genotype examined the intestinal luminal surface for any irregularities and polyps. Regions of tissue with abnormalities were recorded, removed from the surrounding tissue and stored in 10% buffered formalin. Tissue that appeared grossly abnormal was sent for pathologic evaluation. Intestinal polyps were located and diameter measured with the aid of a dissection microscope (Leica, MZ8) equipped with an eyepiece graticule and calibrated to a 50-mm

scale stage micrometer with 0.1 and 0.01-mm graduation. Fourteen-week old $Apc^{Min/mNLS}$ and $Apc^{Min/+}$ mice were analyzed for this study.

Evaluation of proliferation in intestinal epithelia

For proliferation analysis, congenic mice were injected with EdU, and intestinal tissue prepared and stained as described [35]. Briefly, at the time of sacrifice, mouse small and large intestines were removed and opened lengthwise. The colon, and proximal, middle and distal regions of the small intestine were individually rolled as described [36] to form multiple "Swiss Rolls". The sections of rolled colon and small intestine were incubated in fixative [4% paraformaldehyde, 0.1% triton X-100, in PBS] on ice for 1 hour, rinsed in PBS and incubated in 2.5 M sucrose at 4°C overnight. After PBS rinse, rolled tissue sections were frozen in OCT tissue freezing media (VWR) at -20°C. Sections were sliced immediately or stored at -80°C. Tissue cryosections (7μm) were made using a Leica CM1900 cryotome and adhered to glass slides coated with histomount (Invitrogen) for immunohistochemistry. Slides were air dried for approximately 1 min. then tissue slices were permeabilized in 70% methanol before staining for EdU as described [37]. For each tissue in each genotype, 50-100 crypts were analyzed for proliferation by scoring the number of EdU-positive cells and the total cell number by DAPI stain in a crypt cross section. Samples were coded so that analysis could be performed by a trained observer blind to the study parameters. Data from 3 mice of each genotype were collected to determine proliferation in the various intestinal tissues. Only crypts with 40 or more cells in cross section were scored.

Immunohistochemistry of intestinal epithelia and polyps

Tissues were fixed in 10% saline-buffered formalin 16-20 hr then stored in 70% ethanol. Tissues were embedded in paraffin and sectioned at 6-µm. Immunohistochemistry for β-catenin and Ki-67 was performed using the Histomouse kit® (Invitrogen, Cat# 95-9541) according to the manufacturer's protocol and with mouse monoclonal anti- β-catenin antibodies (1:100, BD Biosciences, Cat# 610153) and rat anti mouse Ki-67 antibodies (1:20, Dako, Cat# M7249). Tissues were coded allowing the analysis to be performed by an observer blind to the tissue genotype. Normal appearing Jejunum, Ileum and colon tissues from at least 3 mice of each genotype ($Apc^{+/+}$, $Apc^{mNLS/+}$, and $Apc^{mNLS/mNLS}$) were examined for β-catenin localization. In addition, 6 polyps from 4 $Apc^{Min/+}$ mice and 7 polyps from 3 $Apc^{Min/mNLS}$ mice were examined for β-catenin localization. After surveying the entire length of tissue for β-catenin localization, the average distribution was recorded and representative images captured using 20X and 40X objectives. The polyps were also scored for Ki-67 by counting Ki-67 positive cells per field at 40X magnification. Three fields were photographed and scored for each polyp. The p-values for all proliferation studies were calculated using unpaired, two-tailed t-tests and *GraphPad Prism* software.

Isolation of mouse intestinal epithelial cells:

Isolation of intestinal epithelial cells was performed with modifications to a previous protocol [38]. Immediately after sacrifice, small and large intestines were removed from congenic mice, opened lengthwise and rinsed with cold PBS. Tissue was incubated in 0.04% sodium hypochlorite for 15 min. on ice and then rinsed in cold PBS. The colon and small intestine were then incubated on ice for 15 min in individual 15mL conical tubes containing EDTA/DTT solution (1.5-3mM EDTA and 0.5mM DTT in

PBS). EDTA/DTT solution was poured off and replaced with cold PBS. Tubes were shaken forcefully for 10 sec. to release the epithelial cells from the underlying tissue. The intestinal tissue was removed and placed in a fresh 15mL conical tube of EDTA/DTT solution, and the process was repeated two additional times. The released epithelial cells were collected by centrifugation at 700xg for 5 min. at room temperature. Pellets of the colon epithelia from all three rounds of extraction were resuspended in PBS with protease inhibitors and combined into one sample. Because the surface area of the small intestine is significantly greater than that of the colon, there was no need to combine the epithelial tissue from the replicate extractions. The small intestinal epithelial cells from the second round of extraction were used in the experiments described here. Cell pellets were lysed in Reporter Lysis Buffer (Promega) with protease inhibitors [aprotinin, leupeptin and pepstatin each at 10 μg/ml and 1 mM PMSF] and briefly sonicated. Samples were boiled after addition of Sample Buffer [3X Sample Buffer: 6% w/v Sodium Dodecyl Sulfate, 30% Glycerol, 150mM Tris pH 6.8, ~0.2mg/mL Bromophenol Blue] and resolved by SDS-PAGE before transfer to nitrocellulose membranes.

Immunoblot analysis of Apc and β-catenin in intestinal tissue and MEF fractionation

Immunoblots were processed as described [39]. Membranes were probed with the following primary antibodies diluted as indicated in 5% nonfat dry milk/TBST: rabbit anti-APC M2 (1:3000,[40]), mouse anti-β-catenin (1:2000, BD Biosciences, Cat# 610153), mouse anti-α-tubulin (1:50, Developmental Studies Hybridoma Bank), rabbit anti-fibrillarin (1:1000, ab5821 AbCam), β-actin (1:2000,Sigma). The following secondary antibodies were diluted as indicated: HRP goat anti-mouse (1:10-25,000,

Zymed) and HRP goat anti-rabbit (1:10-25,000, Bio-Rad). Blots were developed using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and a Kodak Image Station 4000R. Band analysis was conducted using Kodak ID Image Analysis Software with protein levels first normalized to β-actin and then values for the $Apc^{+/+}$ samples set to 1. The p-values were calculated using Mann-Whitney nonparametric test. MEFs of each genotype were isolated from congenic (N10-N18) mice on three independent occasions following the protocol as described [41]. Independently isolated early passage MEFs were subjected to fractionation in five independent experiments as described [9]. Nuclear Apc and β-catenin were calculated based on the band intensity in the nuclear fraction divided by the intensity of the nuclear plus the cytoplasmic bands. The p-values were calculated using unpaired, two-tailed t-tests and *GraphPad Prism* software.

Analysis of mRNA using real time RT-PCR

For preparation of RNA, 200-µl of suspended epithelial cells were added to 1-ml Trizol® (invitrogen) and tubes were stored at -80°C until used. RNA extraction was performed using Trizol® according to the manufacturer's protocol. For making cDNA, 1µg total RNA was incubated for 1 hour at 42°C with the cDNA reaction mix containing 1 mM dNTPs, 1µg random hexamer primers (NEB), 1X M-MLuV enzyme buffer (NEB) and 200 units M-MLuV RT enzyme (NEB). The reverse transcriptase enzyme was then inactivated by heating at 95°C for 5 minutes. Quantitative PCR was carried out for *c-Myc*, *Axin2, Cyclin D1, BTEB2, and Hath1* cDNA from the mouse intestinal epithelial cells. The cDNA of the house keeping gene *HGPRT* was used as an internal control. Table 3.1 shows the primers used.

Table 3.1: primers used for quantifying canonical and non-canonical Wnt targets in mouse intestinal epithelial cells

Gene	Forward primer	Reverse primer
c-Myc	5'- TCCTGTACCTCGTCCGATTC-3'	5'- GGTTTGCCTCTTCTCCACAG-3'
Axin2	5'- TGTGAGATCCACGGAAACAG-3'	5'- CTGCGATGCATCTCTCTCTG-3'
Cyclin D1	5'- TTGACTGCCGAGAAGTTGTG-3'	5'- AGGGTGGGTTGGAAATGAAC-3'
Hath1	5'-ACATCTCCCAGATCCCACAG-3'	5'-ACAACGATCACCACAGACCA-3'
BTEB2	5'-CTCCGGAGACGATCTGAAAC-3'	5'-GAACTGGAGGGAGCTGAGG-3'
HGPRT	5'- TGCTCGAGATGTCATGAAGG-3'	5'- TATGTCCCCCGTTGACTGAT-3'

PCR reactions were performed using a DNA engine Opticon 2 system (MJ research) with SYBR green detection system. The total reaction volume was 25µl containing 1X DyNAmo^{TM} HS SYBR$^{®}$ Green qPCR Kit (Finnzymes), 15 picomoles of each primer and 3 µl of 1:2.5 diluted cDNA. Each reaction was done in triplicate and repeated three times. The reaction condition was initial denaturation at 95^{o}C for 15 minutes followed by 40 cycles of denaturation at 94^{o}C for 20 seconds, annealing at 54^{o}C for 30 seconds and extension at 72^{o}C for 30 seconds. The fluorescence was measured at the end of every cycle and a melting curve was analyzed between 40^{o}C and 95^{o}C with 0.2^{o}C increment. Samples were only included in the analysis if the melting curve was a single peak at the expected temperature. Average ΔC(T) was calculated for different genotypes relative to the house keeping gene transcript while ΔΔC(T) of Wnt target cDNA for Apc$^{mNLS/+}$ and Apc$^{mNLS/mNLS}$ was calculated relative to the Apc$^{+/+}$ mice. The p-values were calculated using Mann-Whitney nonparametric test and *GraphPad Prism* software.

Results

Mutation of two Apc NLSs in ES cells and generation of mutant mice

To introduce specific mutations that inactivate both Apc NLSs in mouse embryonic stem (ES) cells and ultimately generate Apc$^{mNLS/mNLS}$ mice, we constructed a gene-targeting vector (Figure 3.1A). The targeted mutations alter a total of six amino

acids in Apc, four in NLS1 and two in NLS2 (Figure 3.1B). These mutations substitute a neutral alanine for the basic lysine residues in the mono-partite NLSs and have been shown to inhibit nuclear import of exogenously expressed APC [19]. Each NLS is adjacent to a SAMP repeat region (so named because they each contain a central Ser-Ala-Met-Pro) which is involved in APC binding to axin. However, mutation of both NLSs does not appear to interfere with axin binding as full-length APC with mutations in both NLSs still interacts with axin (Supplemental Fig. S3.2). These mutations also establish two novel restriction sites, *Sst II* in NLS1 and *Eag I* in NLS2 which were utilized for screening purposes (Figure 3.1B). The targeting vector contains an 11.5 kb genomic *Nhe I/ Not I* fragment encompassing exons 14 and 15 of *Apc* as well as the surrounding introns. The entire *Apc* region of the vector was validated by sequencing. The positive selection marker contained in a germline-induced self-excision cassette was placed in the intron following *Apc* exon 15 [33]. In mice, the tACE promoter initiates transcription of Cre-recombinase during spermatogenesis, inducing Cre-mediated self-excision of the selectable Neo^r marker in the germline of the transgenic animals. Following self-excision, only one 34 bp minimal LoxP element remains in the last *Apc* intron. The targeting construct also contains a copy of the herpes simplex virus thymidine kinase gene (Tk^{HSV}) to allow negative selection of homologous recombinant ES clones.

Identification of homologous recombinant ES clones was accomplished using PCR with different primer pairs inside and outside the targeting construct. Of the 161 ES cell clones that survived positive and negative selection, 107 contained *Apc* with both mutant NLSs (Fig. 3.1C, 3.1D). Eight of these ES cell clones were identified as homologous recombinants by PCR to verify correct insertion of the 3' region of the

vector (Fig. 3.1F, 3.1H). Two were further analyzed by PCR and then subjected to restriction digestion in order to verify correct insertion of the 5' region of the vector and to confirm that both mutant NLSs were integrated (Fig. 3.1F, 1G). One ES line with normal karyotype in 75% of the 50 cells analyzed was used for the initial blastocyst injections. From these injections, 11 chimeric mice, 4 females and 7 males, were obtained; 3 males showed evidence of germline transmission when bred with wild-type C57BL/6J mice.

Elimination of the Neo^r/Cre selection cassette in Apc$^{mNLS/+}$ mice

In the Apc^{1638N} mouse model, a Neo^r selection gene inserted in exon 15 of *Apc* resulted in a dramatic decrease in the expression of the mutant Apc [24, 42]. Thus it was critical to ensure that the Neo^r gene was excised from the Apc$^{mNLS/+}$ mice. Excision of the Neo^r cassette in animals was verified by PCR analysis using oligos within the Neo^r/Cre selection cassette to prime DNA amplification of genomic DNA isolated from Apc$^{mNLS/+}$ mice in the N1 and N2 generations (Figure 3.1E).

Apc$^{mNLS/mNLS}$ mice are viable

Many of the previously generated Apc mouse models display early embryonic lethality as homozygous mutants. When Apc$^{mNLS/+}$ mice were interbred, both Apc$^{mNLS/+}$ and Apc$^{mNLS/mNLS}$ mice were obtained. Thus, the two characterized monopartite nuclear localization signals of Apc are not essential for viability. Apc$^{mNLS/mNLS}$ mice showed no obvious growth defects compared to their wild-type littermates in generations N1-N13. Apc$^{mNLS/mNLS}$ mice are fertile, giving birth to litters of typical size. A long term survival analysis of 55 progeny from first generation Apc$^{mNLS/+}$ females bred to first generation Apc$^{mNLS/+}$ males revealed no significant difference in mouse survival between

$Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ mice (data not shown). Thus, it appears that mutation of *Apc* NLS sequence has limited impact on the lifespan of the mice.

Reduced nuclear Apc in embryonic fibroblasts isolated from $Apc^{mNLS/mNLS}$ mice

Because of complications associated with fractionation of cells obtained from intestinal tissue, we used mouse embryonic fibroblasts (MEFs) isolated from congenic (generation N11) mice to determine effects of Apc^{mNLS} on subcellular Apc localization (Fig. 3.2A). In $Apc^{+/+}$ MEFs, more than one-third of the total Apc was associated with the nuclear fraction (Fig. 3.2B). $Apc^{mNLS/mNLS}$ MEFs were significantly compromised for nuclear Apc distribution, with eleven percent of the total Apc associated with the nuclear fraction. $Apc^{mNLS/+}$ MEFs showed an intermediate phenotype, but nuclear Apc was not significantly different from that in $Apc^{+/+}$ MEFs. β-catenin distribution was similar in all MEF lines (Fig. 3.2C). Moreover, β-catenin appeared predominantly at cell-cell junctions in intestinal tissue from $Apc^{+/+}$, $Apc^{mNLS/+}$ and $Apc^{mNLS/mNLS}$ mice as assessed by immunohistochemistry (Supplemental Fig. S3.3A). A few crypts showed limited nuclear β-catenin staining in cells near their base, but this was consistent for all mouse genotypes (Supplemental Fig. 3.3B).

Apc and β-catenin levels in intestinal tissue

To determine if the single Lox P site remaining in the last *Apc* intron impacts Apc level in Apc^{mNLS} mice, we measured the relative amount of Apc in whole cell protein lysates prepared from epithelial cells isolated from mouse jejunum, ileum and colon (Fig. 3.3). The Apc level did not vary significantly in epithelia from jejunum or ileum tissue isolated from $Apc^{mNLS/mNLS}$, $Apc^{mNLS/+}$ and $Apc^{+/+}$ mice. Unexpectedly, colon epithelia from $Apc^{mNLS/mNLS}$ mice had higher levels of Apc than colon epithelia from $Apc^{+/+}$ mice.

Because Apc targets proto-oncoprotein β-catenin for destruction, higher Apc levels would be expected to result in reduced β-catenin levels. Unexpectedly, colon tissue from $Apc^{mNLS/mNLS}$ mice showed higher levels of β-catenin than colon tissue from $Apc^{+/+}$ mice. In the small intestine, β-catenin levels were not altered in $Apc^{mNLS/mNLS}$ mice compared to $Apc^{+/+}$ mice.

Increased expression of Wnt targets in intestinal epithelia of $Apc^{mNLS/mNLS}$ mice

Based on previous studies performed in cultured cells, we proposed that nuclear APC binds nuclear β-catenin and sequesters it from transcription factor TCF/LEF. Consequently, Wnt target genes activated by the β-catenin/TCF/LEF complex are down-regulated by nuclear APC [11]. Thus we predicted that cells defective in nuclear Apc would be less able to dampen Wnt target gene expression. To test this prediction, mRNA isolated from intestinal epithelial cells was evaluated for relative expression of various Wnt-regulated genes (Figure 3.4). Three genes typically up-regulated in response to a canonical Wnt signal, *c-Myc, Axin2*, and *Cyclin D1* showed higher expression throughout the intestinal epithelia of $Apc^{mNLS/mNLS}$ mice compared to $Apc^{+/+}$ mice. In these same tissues, *Hath 1*, a gene down-regulated in response to a canonical Wnt signal showed lower expression throughout the intestinal epithelia of $Apc^{mNLS/mNLS}$ mice compared to $Apc^{+/+}$ mice. *BTEB2*, a gene reportedly up-regulated in response to non-canonical Wnt signal, showed no expression alterations in jejunum or ileum from $Apc^{mNLS/mNLS}$ mice compared to $Apc^{+/+}$ mice. Unexpectedly, in colon tissue, *BTEB2* expression was higher in both $Apc^{mNLS/mNLS}$ and $Apc^{mNLS/+}$ mice compared to $Apc^{+/+}$ mice.

Increased proliferation in intestinal epithelia of $Apc^{mNLS/mNLS}$ mice

Given the increased expression of *c-Myc* and *Cyclin D1* in intestines of $Apc^{mNLS/mNLS}$ mice, we expected to find an accompanying increase in proliferation. To evaluate epithelial cell proliferation in intestinal tissue, mice were analyzed 4 hours after injection with the thymidine analogue EdU. Crypts from jejunum, ileum and colon each displayed significantly more proliferating cells in $Apc^{mNLS/mNLS}$ mice than in $Apc^{mNLS/+}$ and $Apc^{+/+}$ mice (Fig. 3.5).

Enhanced polyp formation in intestines of $Apc^{Min/mNLS}$ mice

Mice with germline truncating mutations in *Apc* occasionally display a few adenomatous polyps in the colon, with the great majority of polyps found in the small intestine [31]. Therefore, we targeted both colon and small intestine for our initial phenotypic analysis. No adenomatous polyps of the small intestine were identified in any of the $Apc^{mNLS/+}$ mice from generations N1-N4 analyzed for intestinal lesions (n=33). One dysplastic colon polyp was found in an $Apc^{mNLS/mNLS}$ mouse (Fig. 3.6A-D). A polyp was also found in the stomach of another $Apc^{mNLS/mNLS}$ mouse (generation N11).

The few intestinal lesions observed in the $Apc^{mNLS/mNLS}$ mice left open the possibility that nuclear Apc participates in polyp suppression. To examine this more efficiently, congenic $Apc^{mNLS/+}$ mice were bred with $Apc^{Min/+}$ mice. Polyp number, size and distribution were compared in 14-week old progeny $Apc^{Min/+}$ and $Apc^{Min/mNLS}$ mice. Significantly more polyps were found in the jejunum and the ileum of $Apc^{Min/mNLS}$ mice than in $Apc^{Min/+}$ mice (Fig. 3.7A and supplementary Fig S3.4). There were only a few polyps observed in any colon tissue with no significant variation between $Apc^{Min/+}$ and $Apc^{Min/mNLS}$ mice (Fig. 3.7B and supplementary Fig S3.4). Even fewer polyps were observed in the stomach and duodenum tissues, but the average polyp number was

slightly larger in $Apc^{Min/mNLS}$ mice than in $Apc^{Min/+}$ mice (Fig. 3.7B and supplementary Fig S3.4). Combining results from the entire G.I. tissue, we observed nearly twice as many polyps in $Apc^{Min/mNLS}$ mice than in $Apc^{Min/+}$ mice (Fig. 3.7C). These results suggest that the Apc^{mNLS} enhances polyp initiation or development in the Apc^{Min} mouse model. Furthermore, the average polyp size was significantly larger in jejunum and ileum tissue of $Apc^{Min/mNLS}$ mice compared to $Apc^{Min/+}$ mice (Fig. 3.7D). Collectively, the results from this study suggest that nuclear Apc contributes to a tumor suppressor phenotype.

Increased proliferation in intestinal polyps from $Apc^{Min/mNLS}$ mice

To begin to explore the mechanism of enhanced polyp formation in $Apc^{Min/mNLS}$ mice, we examined cellular proliferation and β-catenin distribution in polyps from $Apc^{Min/mNLS}$ and $Apc^{Min/+}$ mice. Strong nuclear β-catenin staining was observed in polyps from both $Apc^{Min/mNLS}$ and $Apc^{Min/+}$ mice (Fig. 3.7F, a-d). In contrast, β-catenin appeared predominantly at the cell-cell junctions of normal intestinal cells from both $Apc^{Min/mNLS}$ and $Apc^{Min/+}$ mice. Although nuclear β-catenin was prevalent in both $Apc^{Min/mNLS}$ and $Apc^{Min/+}$ polyps, not every cell with nuclear β-catenin was positive for proliferation marker Ki-67 (Fig. 3.7F, e-h). Closer examination of Ki-67-positive cells revealed that polyps from $Apc^{Min/mNLS}$ mice had significantly more proliferating cells than polyps from $Apc^{Min/+}$ mice (Fig. 3.7E). Combined with the observation that there were more polyps and these polyps were larger in intestines of the $Apc^{Min/mNLS}$ mice than in the $Apc^{Min/+}$ mice (Fig. 3.7 A-D), it is likely that contributions of nuclear Apc to regulation of cellular proliferation impact tumor suppression in this newly developed model.

Discussion

Nuclear APC has been observed in cultured cells, in cells of various model organisms and in human tissues [43]. Roles for nuclear APC in DNA repair and replication as well as Wnt signal regulation have been proposed [10, 12, 14-16, 44]. However, to date, experimental manipulation of nuclear APC has been confined to cultured cells. In this report, we describe a new mouse model with mutations "knocked-in" the *Apc* gene which inactivate the two canonical Apc NLSs [19]. To our knowledge, the $Apc^{mNLS/mNLS}$ model is the first mouse generated to specifically inhibit nuclear import of any protein by knock-in mutations that target and inactivate an NLS. $Apc^{mNLS/mNLS}$ mice were viable and displayed increased proliferation of epithelia throughout the small intestine and colon. Epithelia from jejunum, ileum, and colon also exhibited increased canonical Wnt signaling as evidenced by increased expression of genes up-regulated by canonical Wnt signal (*cyclin D1, c-myc and Axin 2*) and decreased expression of genes down-regulated by canonical Wnt signal (*Hath 1*). This increased Wnt signaling could explain the enhanced proliferation observed in these same tissues. Although only occasionally observed in $Apc^{mNLS/mNLS}$ mice, polyps in jejunum and ileum were significantly more abundant and larger, with increased proliferative index in $Apc^{Min/mNLS}$ mice compared to $Apc^{Min/+}$ mice, implicating nuclear Apc in suppression of polyp formation.

The best characterized role for nuclear APC involves negative regulation of Wnt signaling [10, 14]. Based on studies performed in cultured cells, it was proposed that nuclear APC binds to nuclear β-catenin, sequestering it from a complex with activated TCF/LEF [10] and mediating β-catenin export to the cytoplasm [11-13]. There is also evidence that APC can interact with transcriptional corepressor CtBP [14], resulting in

inhibition of TCF/LEF-mediated transcription and dampening of a Wnt signal [45]. Our data indicate that Wnt signaling is up-regulated in $Apc^{mNLS/mNLS}$ mice, supporting a role for nuclear Apc as a negative regulator of Wnt signaling. Of note, in this study, the alterations in Wnt gene expression occurred without increased β-catenin levels. This finding supports an alternate mechanism that does not require destruction of β-catenin for Apc to elicit dampening of a Wnt signal. We also found that cells from $Apc^{mNLS/mNLS}$ mice had no increase in nuclear β-catenin, consistent with a role for nuclear Apc in transport of transcription repressors to the β-catenin/TCF/LEF complex and/or sequestration of nuclear β-catenin. However, as cells from $Apc^{mNLS/mNLS}$ mice displayed some nuclear Apc, we can not fully exclude the possibility that Apc also plays a role in nuclear export of β-catenin.

Some Wnt target genes such as c-Myc and cyclin D1 are associated with cell cycle progression [46, 47]. If nuclear Apc down-regulates Wnt signaling, then reduced nuclear Apc should lead to enhanced TCF/LEF-mediated transcription, increased expression of c-Myc and cyclin D1, and consequently, more proliferation. Our finding that intestinal epithelia of $Apc^{mNLS/mNLS}$ mice display increased Wnt signaling, including up-regulation of c-Myc and cyclin D1, and increased proliferation further supports a role for nuclear Apc in control of cell cycle progression.

The mutations introduced into *Apc* were minimal, and we expected the phenotype of $Apc^{mNLS/mNLS}$ mice would be more subtle than that of previous *Apc* mouse models which express truncated Apc proteins. The combined six amino acid substitutions in Apc NLS1 and NLS2 do not appear to impact Apc binding to β-catenin or axin (supplemental Fig S3.2). Apc and β-catenin levels in epithelia isolated from either jejunum or ileum

were similar in $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ mice (Fig. 3.3). Therefore, the alteration in expression of canonical Wnt targets seen in these tissues did not likely result from compromised β-catenin degradation and it is likely that the observed phenotypes of $Apc^{mNLS/mNLS}$ mice result from compromised nuclear import of Apc.

Nuclear Apc entry was severely compromised, but not entirely eliminated in MEFs from $Apc^{mNLS/mNLS}$ mice (Fig. 3.2). The two Apc NLSs that were inactivated in our mouse model are each classic monopartite NLSs, which bind to importin-α in the cytoplasm to target protein transport through the nuclear pore [48, 49]. The continued presence of some Apc in the nuclei of MEFs isolated from $Apc^{mNLS/mNLS}$ mice supports the existence of alternative processes by which Apc may gain nuclear access. In the mouse model described here, the two canonical NLSs are eliminated, but the Armadillo repeat domain which has been previously implicated in nuclear import of APC remains intact [20]. In human polyp tissue and colon cancer cell lines, the Armadillo repeat domain has been proposed to mediate nuclear entry of truncated forms of APC that lack the canonical NLSs [7, 50]. Moreover, the 15-amino acid repeat region of APC (aa. 959-1338) can facilitate nuclear import of a fused green fluorescent protein [39]. It is possible that one or both of these auxiliary Apc domains binds to nuclear import machinery directly, or may allow Apc binding to other proteins that are able to facilitate nuclear import or retention of Apc.

The most surprising result from this study was that $Apc^{Min/mNLS}$ mice had nearly twice as many polyps as $Apc^{Min/+}$ mice (Fig. 3.7C). Furthermore, polyps from $Apc^{Min/mNLS}$ mice were larger on average than those from $Apc^{Min/+}$ mice (Fig. 3.7D). Together, these observations are consistent with a role for nuclear Apc in suppression of polyp initiation

or progression. $Apc^{mNLS/mNLS}$ mice showed increased proliferation and Wnt signaling, either of which would be expected to enhance polyp formation. A recent report showed an association of Wnt target gene up-regulation with increased intestinal adenomas in $Apc^{1322T/+}$ mice [51]. It is also possible that the observed increase in polyp number and size might result from a role for nuclear Apc in suppressing polyp progression, once the polyp is initiated. In this scenario, polyps would initiate at the same rate in $Apc^{Min/mNLS}$ and $Apc^{Min/+}$ mice, but would more rapidly grow large enough for detection in the $Apc^{Min/mNLS}$ mice. Our finding of increased proliferation in polyps from $Apc^{Min/mNLS}$ mice compared to $Apc^{Min/+}$ mice does not distinguish between these two scenarios, but does implicate nuclear Apc regulation of cell proliferation as a contributing factor to tumor suppression.

In the current study, three separate parameters each showed a response in the colon distinct from that seen in the jejunum or ileum. Epithelia from jejunum or ileum did not display elevated levels of either Apc or β-catenin (Fig. 3.3). In contrast, colon epithelia displayed Apc and β-catenin protein levels that were significantly higher in $Apc^{mNLS/mNLS}$ mice compared to $Apc^{+/+}$ mice. This finding confirms that high levels of Apc do not always result in constitutive β-catenin degradation. Changes in expression of Wnt target genes were consistent with elevated canonical Wnt signaling in jejunum, ileum and colon tissues from $Apc^{mNLS/mNLS}$ mice compared to $Apc^{+/+}$ mice (Fig. 3.4). However, BTEB2, a gene not regulated by canonical Wnt signaling, was unchanged in jejunum and ileum, but was elevated in colonic epithelial cells from $Apc^{mNLS/mNLS}$ mice compared to $Apc^{+/+}$ mice. Finally, unlike small intestinal tissue, colon tissue from $Apc^{Min/mNLS}$ mice did not show elevated polyp size or multiplicity compared to $Apc^{Min/+}$

mice (Fig. 3.7B, 3.7D). Why do the colon and small intestine tissues show distinct responses to manipulation of Apc NLSs? We speculate that this variability might result from potential alternate feedback responses (chromosomal instability, apoptosis, survival) dependent on tissue type. Moreover, colon tissue lacks villi, performs a distinct function and maintains a different flora than the small intestine. Although not completely understood, humans and rats with germline *APC* mutations are prone to colon polyps, whereas mice with similar *Apc* mutations have polyps predominantly in the small intestine. Any of these factors might contribute to the differences seen in colon and small intestinal tissue from Apc^{mNLS} mice. Future studies utilizing the novel $Apc^{mNLS/mNLS}$ model might offer clues to some of these longstanding puzzles in Apc biology.

In summary, the novel $Apc^{mNLS/mNLS}$ mouse model contains a subtle alteration that inactivates both Apc NLSs. Increased expression of Wnt targets and increased proliferation in intestines of adult $Apc^{mNLS/mNLS}$ mice implicate nuclear Apc in control of cellular proliferation and Wnt signaling. The finding of increased numbers and size of small intestinal polyps in $Apc^{Min/mNLS}$ mice compared with $Apc^{Min/+}$ mice implicates nuclear Apc in tumor suppression. Future studies of this novel mouse model will elucidate nuclear Apc contributions to other cellular events critical for tissue homeostasis, such as DNA repair, transcription regulation and cell cycle progression.

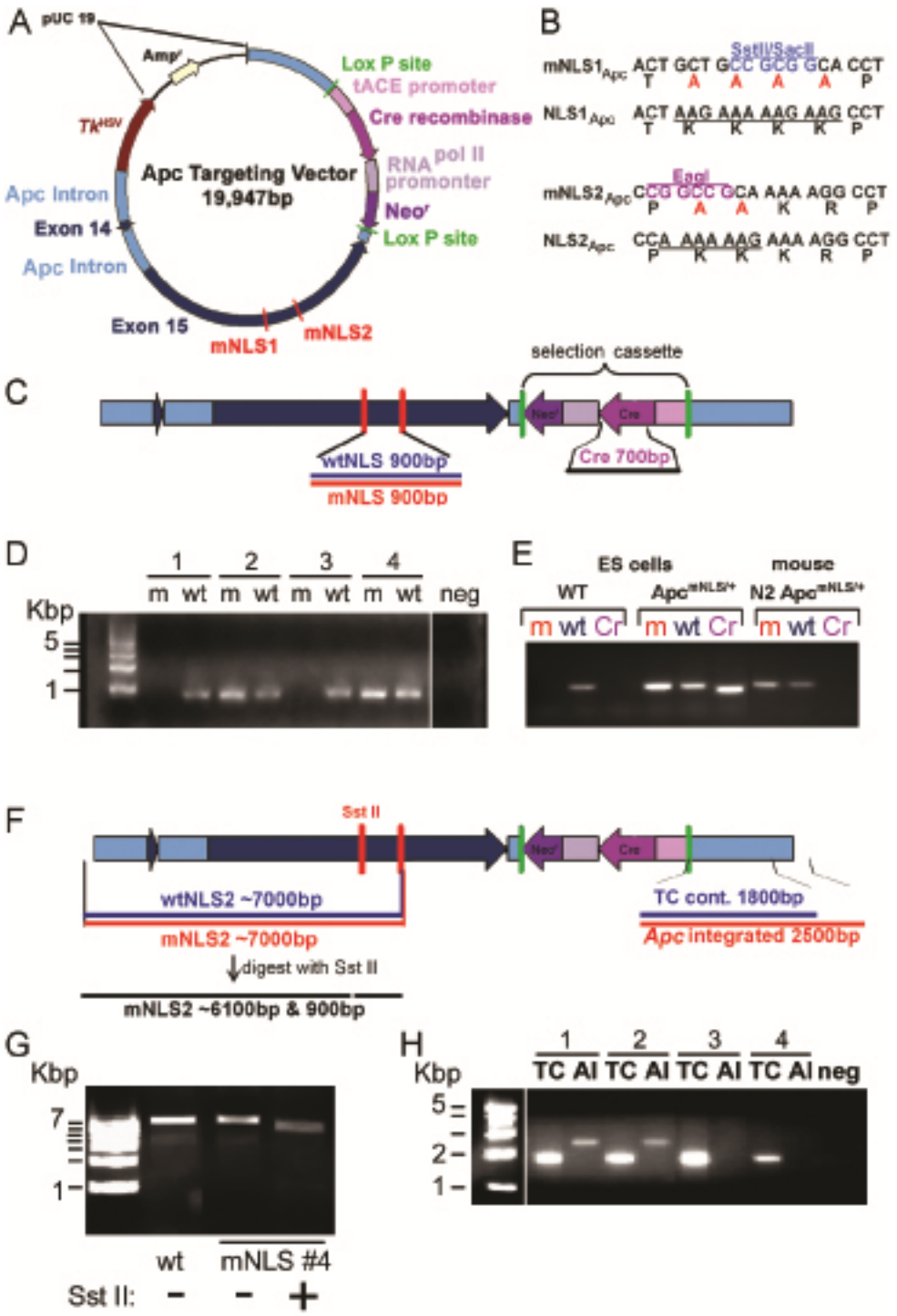


Figure 3.1. Generation of Apc$^{mNLS/+}$ mouse ES cell lines. (A) Schematic representation of Apc NLS targeting vector. Drawn roughly to scale, the vector includes *Apc* exons 14 and 15 (dark blue), with surrounding introns (light blue). A Neomycin resistance gene (Neo^r, purple) inserted in the final *Apc* intron is flanked by Lox P sites (green) that will facilitate its excision when expressed in the testes where the tACE promoter (murine angiotensin-converting enzyme) drives expression of Cre recombinase. The herpes

simplex virus thymidine kinase gene (Tk^{HSV}) allows negative selection of homologous recombinant ES cell clones. **(B)** Mutations in Apc NLS1 and NLS2 result in amino acid substitutions inactivating the nuclear localization signals and also introduce unique restriction enzyme sites for screening purposes. **(C)** Analysis of ES cell lines with PCR using primers specific for the wildtype (wt) or mutant (m) Apc NLS allowed identification of 107 lines with both Apc NLS1 and NLS2 mutated. **(D)** Samples 2 and 4 in the gel image shown each have both Apc NLSs mutated. PCR reaction without template DNA (neg). **(E)** Excision of the Ace-Cre-Neo^r selection cassette was verified in N2 $Apc^{mNLS/+}$ mice using PCR with primers specific for Cre. Genomic DNA isolated from wild type (WT) and $Apc^{mNLS/+}$ ES cell lines was used as a negative and positive control, respectively, for the Cre primer PCR (Cr). PCR using primers specific for wild type sequence (wt) and mutant sequence (m) confirmed that the mouse analyzed was $Apc^{mNLS/+}$ and demonstrated genomic DNA quality sufficient for PCR analysis. **(F)** Scheme to determine correct integration of the 5' and 3' ends of the targeting vector. **(G)** Correct integration of the 5' end of the targeting vector was established by PCR analysis using a primer specific for mutant *Apc* NLS2 with a second primer that recognizes genomic sequence outside the targeting construct and upstream of a correctly integrated vector. Products of this PCR reaction were digested with *Sst II* to confirm integration of mutant Apc NLS1. **(H)** PCR analysis using a primer unique to the targeting construct with a second primer that recognizes genomic sequence outside the targeting construct and downstream of a properly integrated vector allowed identification of 8 recombinant ES cell lines that had correctly integrated the 3' end of the targeting vector (samples 1 and 2). TC, targeting vector control product is ~ 1800 bp if targeting vector is integrated into genomic DNA. AI, *Apc* integrated product is ~ 2500 bp if targeting vector is properly inserted into the *Apc* gene.

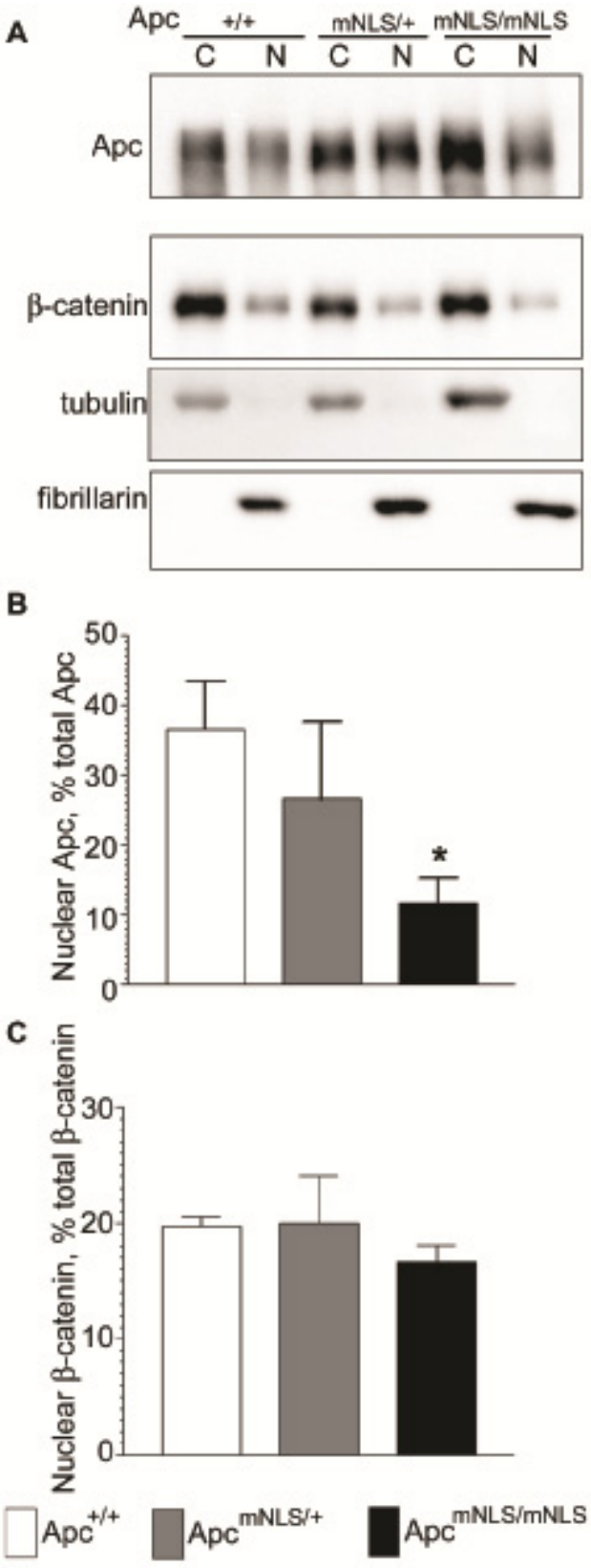

A
Apc
+/+
mNLS/+
mNLS/mNLS
C
N
C
N
C
N
Apc
β-catenin
tubulin
fibrillarin
B
Nuclear Apc, % total Apc
50
40
30
20
10
0
*
C
Nuclear β-catenin, % total β-catenin
30
20
10
0
Apc+/+
ApcmNLS/+
ApcmNLS/mNLS

Figure 3.2. Reduced nuclear Apc levels in MEFs from $Apc^{mNLS/mNLS}$ mice.
(A) Mouse Embryonic Fibroblasts (MEFs) isolated from congenic $Apc^{+/+}$, $Apc^{mNLS/+}$ and $Apc^{mNLS/mNLS}$ mice were subjected to fractionation followed by immunoblot. A single blot, probed for Apc, β-catenin, tubulin (cytoplasmic marker) and fibrillarin (nuclear marker) is shown. **(B)** Apc band intensities were determined and results from five independent fractionation experiments performed on three different isolations of MEF cells are presented as the fraction of the total Apc protein in the nucleus ± SEM. $Apc^{mNLS/mNLS}$ MEFs displayed significantly less nuclear Apc than $Apc^{+/+}$ MEFs ($p = 0.012$). **(C)** β-catenin distribution, determined as in (B), was similar in all MEF lines.

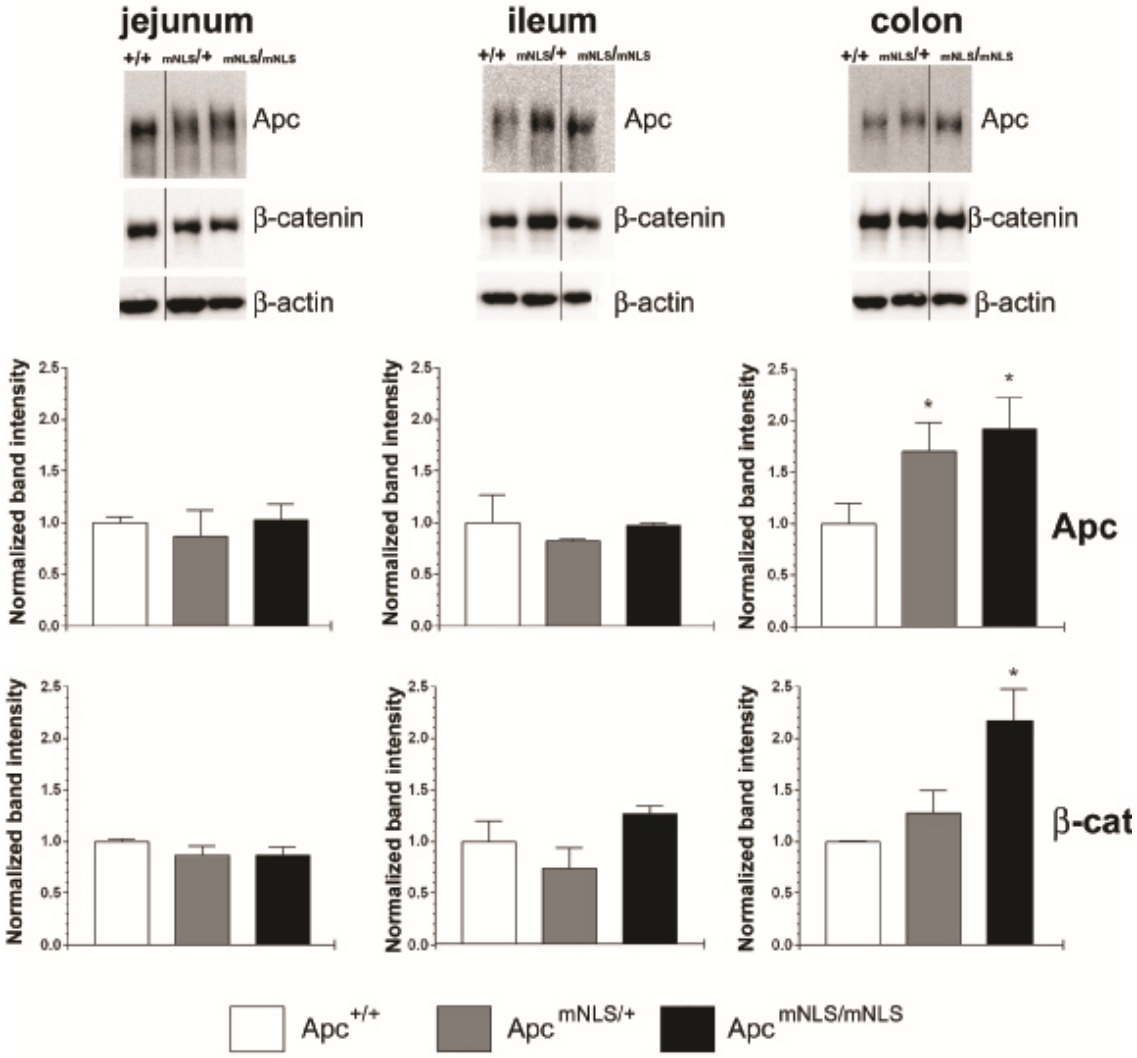


Figure 3.3. Apc and β-catenin levels in intestinal epithelia from Apc$^{mNLS/+}$ and Apc$^{mNLS/mNLS}$ mice. Epithelial cells were isolated from three intestinal segments (jejunum, ileum and colon) of congenic Apc$^{+/+}$, Apc$^{mNLS/+}$ and Apc$^{mNLS/mNLS}$ mice. Proteins from whole cell lysates were resolved using SDS-PAGE. Immunoblots were probed for Apc, β-catenin and β-actin which served as a loading control (top panels). Band intensities were determined for four samples from each genotype and are presented as average band intensity ± SEM relative to β-actin and normalized to the Apc$^{+/+}$ sample which was set to 1 (middle and bottom panels). Apc and β-catenin levels appeared comparable in epithelial cells isolated from jejunum and ileum of mice of each genotype. Using the Mann-Whitney nonparametric test, colon tissue from Apc$^{mNLS/mNLS}$ mice showed significantly higher levels of both Apc ($p = 0.017$) and β-catenin ($p = 0.01$) than colon tissue from Apc$^{+/+}$ mice.

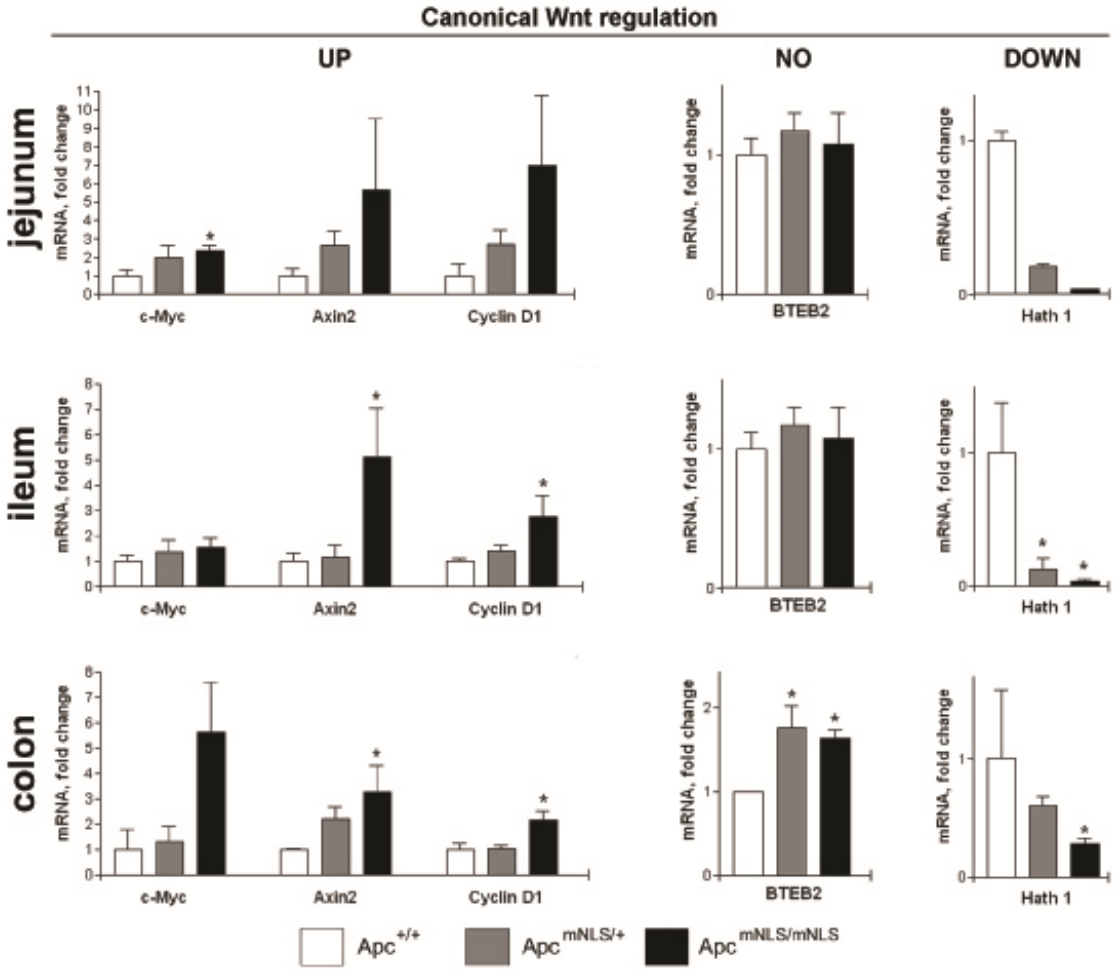


Figure 3.4. Elevation of Wnt target gene expression in Apc$^{mNLS/mNLS}$ mice. Epithelial cells were isolated from three intestinal segments (jejunum, ileum and colon) of congenic Apc$^{+/+}$, Apc$^{mNLS/+}$ and Apc$^{mNLS/mNLS}$ mice. For each sample, mRNA levels of three genes up-regulated by canonical Wnt signaling (*cMyc, Axin2*, and *Cyclin D1*), one gene which is down-regulated by canonical Wnt signaling (Hath1), and one gene which is not regulated by canonical Wnt signaling (*BTEB2*), each normalized to *HGPRT* (housekeeping gene, control) were determined using real time quantitative RT-PCR. Results from 4-6 mice are presented as average mRNA level relative to that found in the Apc$^{+/+}$ sample ± SEM. p values < 0.05 as calculated using the Mann-Whitney nonparametric test, are indicated with *. Levels of *Hath 1* mRNA in jejunum samples from Apc$^{mNLS/mNLS}$ mice were around the lower limit of detection, precluding calculation of a $\Delta\Delta C(T)$ for some samples.

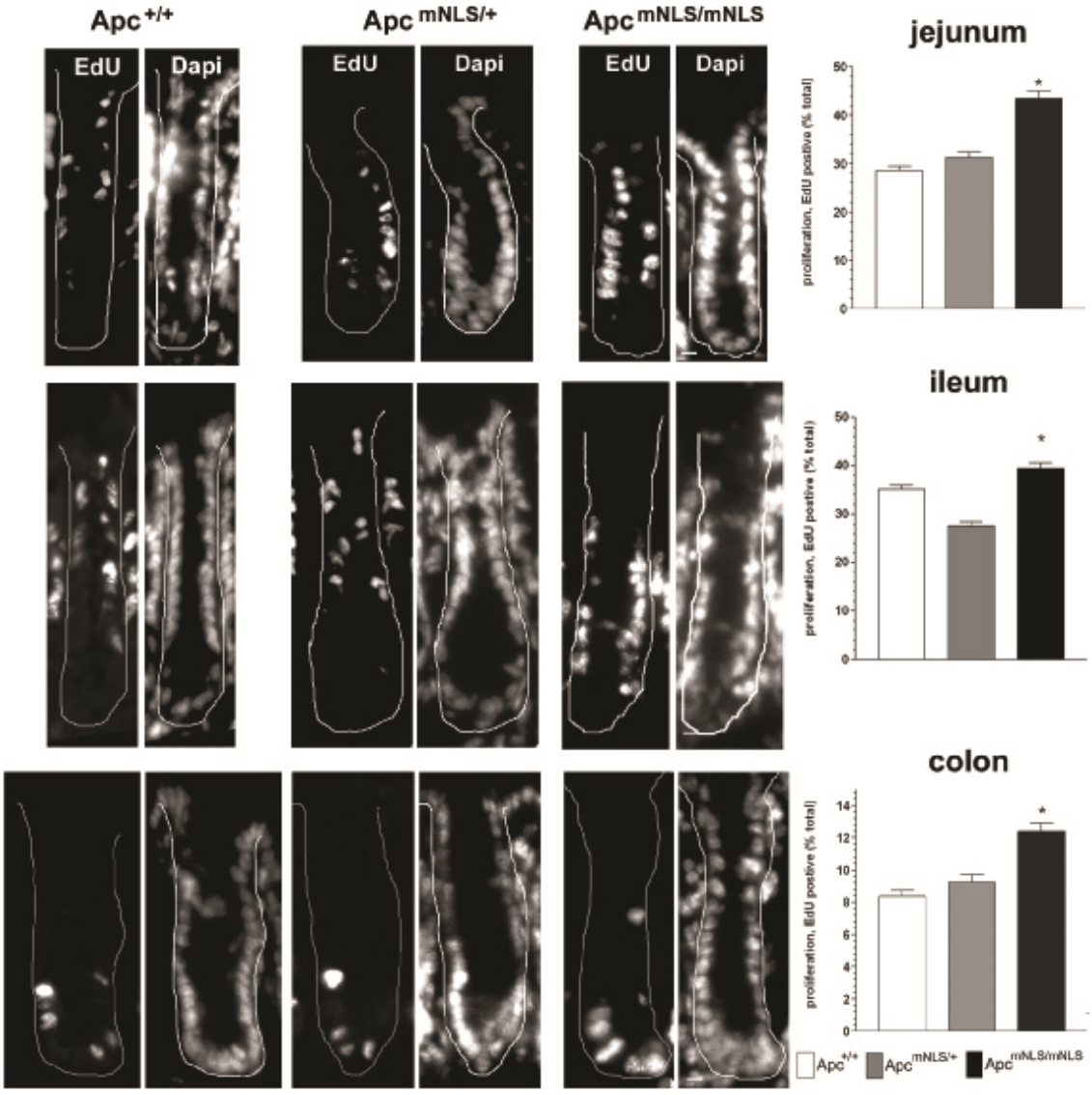


Figure 3.5. Increased epithelial cell proliferation in intestines of Apc$^{mNLS/mNLS}$ mice. **R**epresentative images of crypts from EdU-labeled intestinal tissue isolated from Apc$^{+/+}$, Apc$^{mNLS/+}$ and Apc$^{mNLS/mNLS}$ mice with white line denoting crypt border (left panels). Dapi staining allowed identification of cell nuclei. Scale bar, 10 μm. Right panels, the average number of EdU-positive cells per crypt cross section normalized to the total crypt cell number is presented with error bars indicating SEM. Samples were collected from 3 mice of each genotype. **Top,** jejunum tissues showed a significant increase in proliferation in Apc$^{mNLS/mNLS}$ mice compared to Apc$^{+/+}$ mice ($p < 0.0001$). **Middle,** ileum tissues showed a significant increase in proliferation in Apc$^{mNLS/mNLS}$ mice compared to Apc$^{+/+}$ mice ($p < 0.05$). **Bottom,** colon tissues showed a significant increase in proliferation in Apc$^{mNLS/mNLS}$ mice compared to Apc$^{+/+}$ mice ($p < 0.0001$).

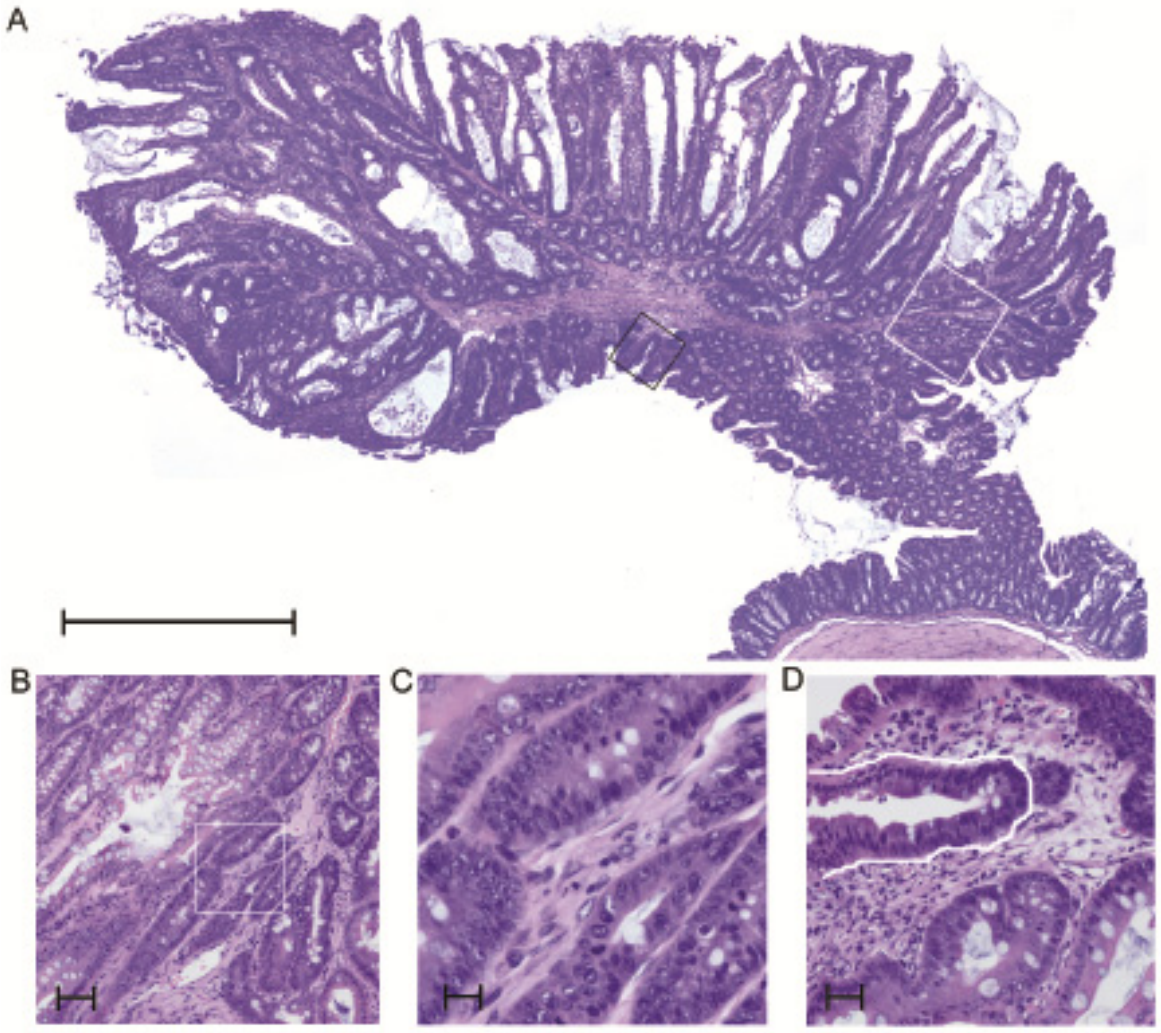


Figure 3.6. Polyps in Apc$^{mNLS/mNLS}$ mice. (A) Low power magnification of lesion in colon of Apc$^{mNLS/mNLS}$ mouse. scale bar, 1 mm. **(B)** Same lesion with higher magnification of region outlined by white box in (A) showing epithelial atypia. Nuclei are enlarged with loss of polarization. scale bar, 100 μm. **(C)** Higher power magnification of region with cell atypia from (B) showing enlarged nuclei. scale bar, 25 μm. **(D)** A progression of atypia is shown in the same tissue (approximate area shown in black box in (A)) from lower right with more polarized nuclei to upper left with enlarged non-polarized nuclei. A representative dysplastic crypt is outlined in white. scale bar, 50 μm.

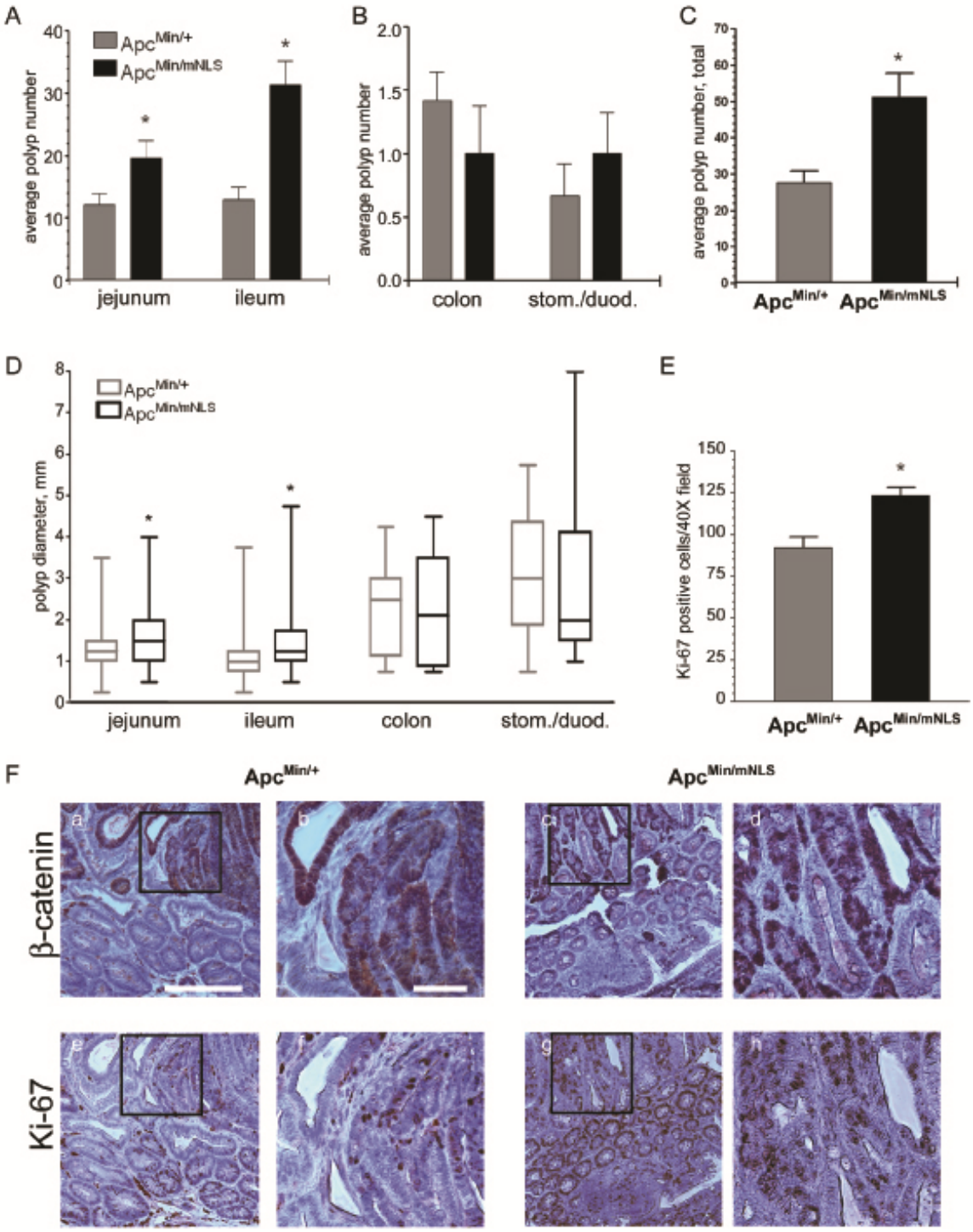
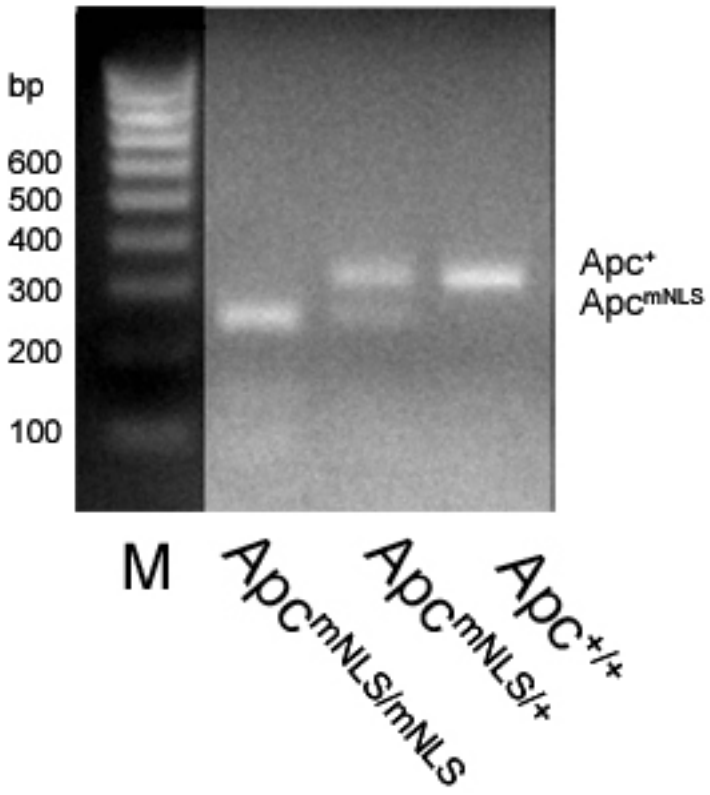
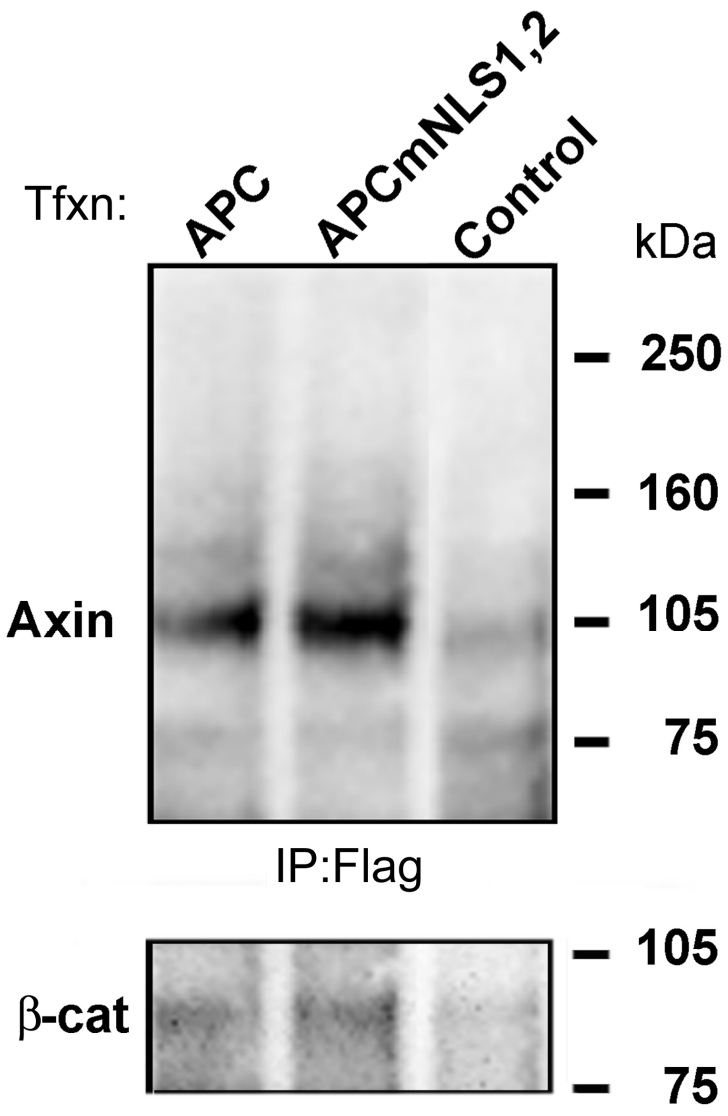

A
B
C
D
E
F
average polyp number
jejunum
ileum
colon
stom./duod.
average polyp number, total
polyp diameter, mm
Ki-67 positive cells/40X field
β-catenin
Ki-67

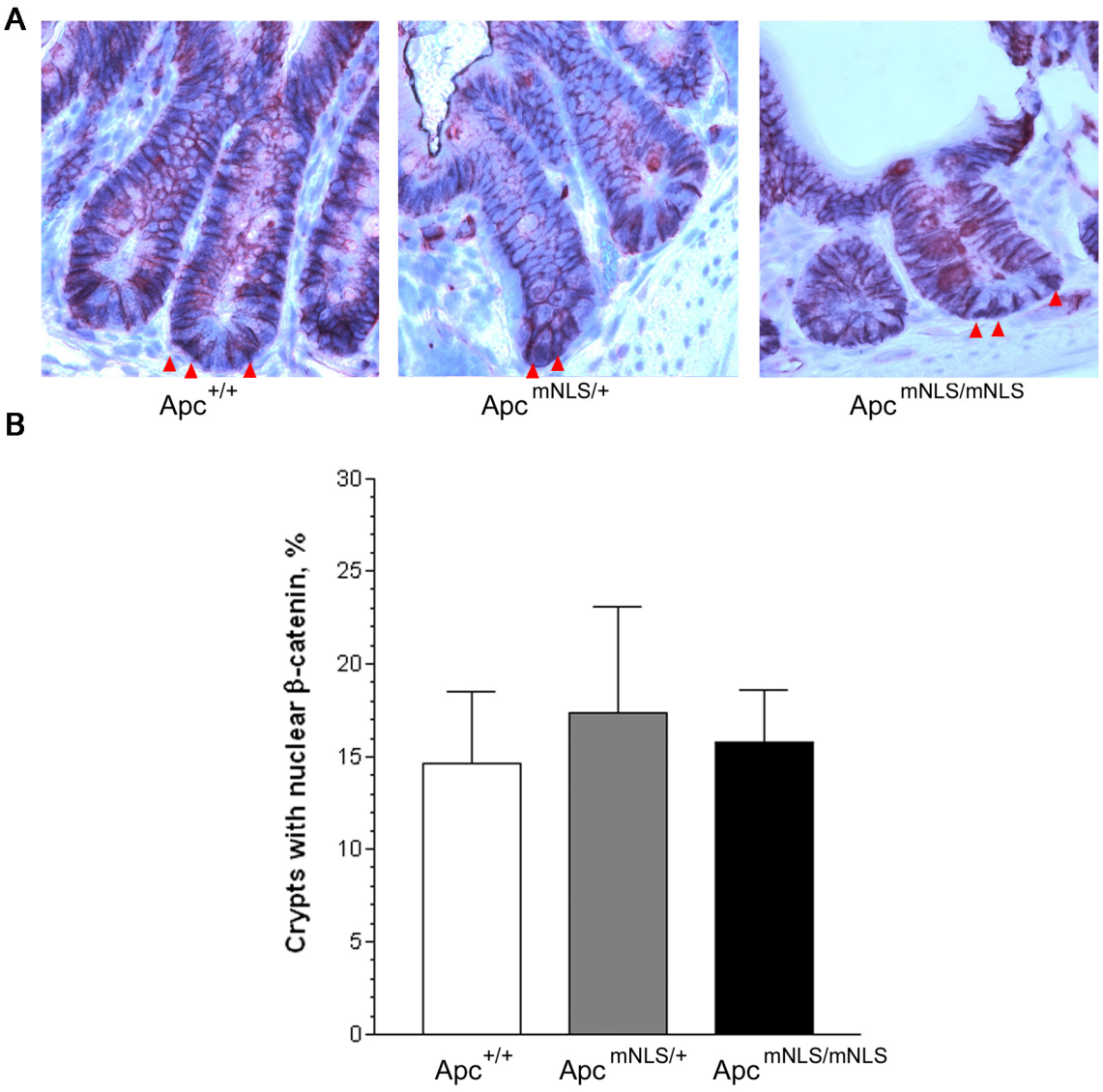
Figure 3.7. $Apc^{Min/mNLS}$ mice have more and larger polyps than $Apc^{Min/+}$ mice. Polyps were identified and measured in fourteen-week old $Apc^{Min/+}$ (n=12) and $Apc^{Min/mNLS}$ (n=8) mice. **(A)** Results presented as average polyp number per mouse ± SEM indicate significantly more polyps in the jejunum ($p = 0.035$) and the ileum ($p = 0.0002$) of $Apc^{Min/mNLS}$ mice compared to $Apc^{Min/+}$ mice. **(B)** Polyps were also identified in the colon, stomach, and duodenum but showed no statistically significant differences in $Apc^{Min/mNLS}$ and $Apc^{Min/+}$ mice. **(C)** Results from all G.I. tissues combined (jejunum, ileum, colon, stomach, duodenum) indicate significantly more polyps in $Apc^{Min/mNLS}$ compared to $Apc^{Min/+}$ mice ($p = 0.0021$). **(D)** Diameters of all polyps are shown as a box and whisker plot. Polyps in both jejunum and ileum were significantly larger in $Apc^{Min/mNLS}$ mice than in $Apc^{Min/+}$ mice ($p < 0.0001$). **(E)** Significantly more proliferation, as determined by Ki-67 expression, was observed in polyps isolated from the ileums of $Apc^{Min/mNLS}$ mice than $Apc^{Min/+}$ mice ($p = 0.0003$). (F) A tissue section of polyp from $Apc^{Min/+}$ or $Apc^{Min/mNLS}$ mice stained for β-catenin (a-d) with subsequent section stained for Ki-67 (e-h). Scale bar in (a) is 200 μm and also corresponds to (c), (e), and (g). Panels (b) (d) (f) and (h) show higher power magnifications of regions indicated with black boxes in the previous panels, with 50 μm scale bar shown in (b).



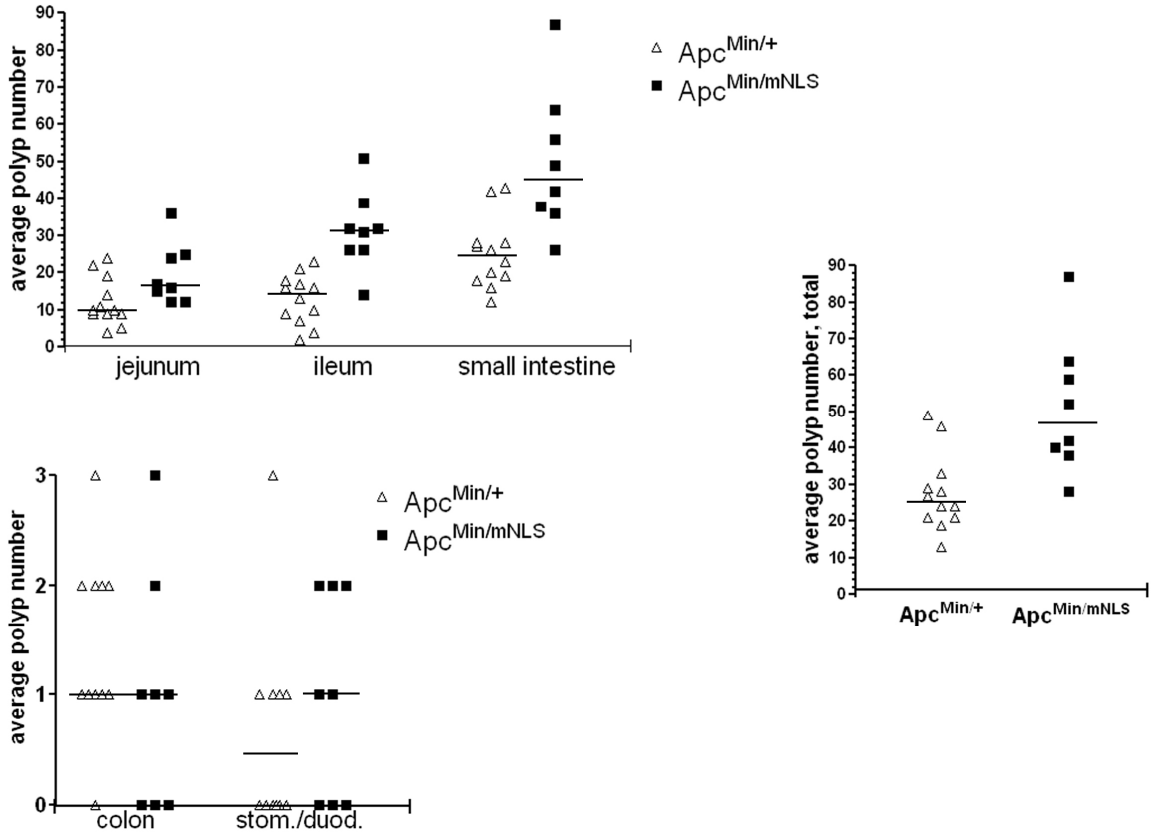
Supplemental Figure S3.1. Genotyping Apc$^{mNLS/mNLS}$ mice. Genomic DNA from congenic mice was amplified by PCR using the primers described in Materials and Methods. Product DNA was cut with *Sac*II restriction enzyme to distinguish *Apc^{mNLS}* from *Apc^{+}*.



Supplemental Figure S3.2. Mutations in APC NLS1 and NLS2 do not interfere with axin or β-catenin binding. 293T cells were transfected with expression constructs for flag-tagged full-length APC or APC with mutations in both NLS1 and NLS2 (APCmNLS1,2). Anti-flag antibody was used to precipitate protein from transfected cells (Tfxn) or control untransfected cells. Precipitated proteins were analyzed for the presence of axin and β-catenin by immunoblot



Supplemental Figure S3.3. β-catenin distribution similar in intestinal epithelia from $Apc^{+/+}$, $Apc^{mNLS/+}$, and $Apc^{mNLS/mNLS}$ mice. (A) At least three mice from each genotype were analyzed for β-catenin localization. In all normal intestinal tissues, β-catenin appeared predominantly at the cell-cell junctions, with occasional nuclear distribution in cells near the crypt base (red arrows). **(B)** Determination of crypts that contained any cells with nuclear β-catenin revealed a similar number in mice from each genotype (p=0.826).



Supplemental Figure S3.4. $Apc^{Min/mNLS}$ mice have more polyps than $Apc^{Min/+}$ mice. Polyps identified in 14-week old mice (see Fig. 7) are presented as scatter plots. Lines indicate the sample median.

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Chapter 4

The data and opinions in this chapter were submitted for publication in "*Gastroenterology"* and reformatted for this dissertation

Nuclear Adenomatous polyposis coli suppresses colitis-associated tumorigenesis in mice

Abstract

Because mutation of tumor suppressor gene *APC* is the initiating step in most colorectal cancers (CRC), understanding the full spectrum of APC functions will illuminate better diagnostic, preventive and therapeutic strategies for the disease. Although APC shuttles between the cytoplasm and nucleus, testing proposed roles for nuclear APC in the context of a whole organism was only recently made possible using a mouse model compromised for nuclear Apc which we generated by introducing germline mutations that inactivate the Apc nuclear localization signals (Apc^{mNLS}). Our previous analysis of Apc^{mNLS} mice revealed a role for nuclear Apc in regulation of Wnt signal transduction and intestinal cell proliferation as well as in tumor suppression. In humans, chronic colitis significantly increases CRC risk and *APC* mutations occur late in this cancer progression. In the current study, we show increased expression of inflammatory mediators cyclo-oxygenase-2 (*Cox-2*) and macrophage-inflammatory-protein-2 (*MIP-2*) in colon epithelial cells from $Apc^{mNLS/mNLS}$ mice, suggesting a role for nuclear Apc in suppressing colitis-mediated colon cancer. To test this hypothesis, we initiated colon tumors with a single injection of the mutagen, azoxymethane (AOM) and promoted the tumors with repeated oral administration of dextran sodium sulfate (DSS) to induce colonic inflammation. When treated with AOM-DSS, $Apc^{mNLS/mNLS}$ mice developed more colonic tumors than treated wildtype mice. Tumors from treated $Apc^{mNLS/mNLS}$ and wildtype mice had the same spectrum of β-catenin mutations, proliferation rates and histopathological features, consistent with the Apc^{mNLS} allele enhancing colitis-associated tumor initiation rather than progression. $Apc^{mNLS/mNLS}$ mice had increased weight loss and colonic lymphoid follicles implicating nuclear

Apc in suppression of AOM-DSS-induced colitis. These findings reveal novel functions for nuclear Apc and also indicate a critical protective role for Apc *early* in inflammation-induced colon tumorigenesis.

Introduction

As the second leading cause of cancer-related deaths in the United States, colorectal cancer (CRC) is responsible for nearly 50,000 deaths each year. Inflammation is considered a major risk factor for CRC and one fifth of all persons with inflammatory bowel disease (IBD) develop colon cancer. This colitis-associated cancer is characterized by poor prognosis and relatively high mortality rate of ~50% [1]. Anti-inflammatory drugs such as Aspirin and Celebrex can reduce the risk of CRC, not only in persons with IBD but also in the general population [2]. A better understanding of the contribution of inflammation to the underlying biology of CRC is needed to develop more effective preventive, diagnostic and therapeutic regimens.

Adenomatous polyposis coli (APC) is the primary CRC suppressor gene, with APC mutations implicated in initiation of >80% of all CRC. The *APC* gene product is a large (310 kDa) multi-domain protein, which localizes to both the cytoplasm and the nucleus [3]. APC has many cellular functions, the best characterized of which is antagonizing the Wnt signaling pathway [4]. In this capacity, APC forms a cytoplasmic complex with axin, GSK3β, and other proteins that targets the oncoprotein β-catenin for proteasome-mediated degradation [5]. APC also shuttles between the cytoplasm and the nucleus using at least 2 nuclear localization signals (NLS) and 5 nuclear export signals (NES) [6].

APC has several proposed nuclear functions. APC and β-catenin can interact in the nucleus [7] and we provided evidence that this interaction leads to transcriptional repression of Wnt target genes and ultimately inhibition of cellular proliferation [6]. We also showed that

nuclear APC interacts with Topoisomerase IIα, a critical enzyme required for DNA replication and a target for traditional cancer chemotherapeutics [8]. Studies from other groups implicate nuclear APC in DNA repair [9] and synthesis [10]. Until recently, analyses of nuclear APC activities relied on purified proteins or cultured cells as there was no animal model to enable characterization of nuclear APC functions in the context of a whole organism.

To better understand the role of nuclear APC in tissue homeostasis and tumor suppression, we generated a mouse model in which the nuclear import of Apc is compromised by the introduction of mutations into both NLSs [11]. We found that these mutant mice ($Apc^{mNLS/mNLS}$) have higher rates of cellular proliferation than their wild-type counterparts and increased expression of Wnt target genes [11]. We also found that $Apc^{Min/+}$ mice, the most widely utilized mouse model of intestinal polyposis mediated by truncating germline *Apc* mutation, develop more and larger intestinal tumors when they also harbor the Apc^{mNLS} allele ($Apc^{mNLS/Min}$). Together these data suggest a tumor suppressor function for nuclear Apc [11].

In the current study, we tested the potential roles for nuclear Apc in the AOM-DSS mouse model of colitis-mediated colon cancer. In this model, colon cancers are initiated with a single injection of the mutagen, azoxymethane (AOM) and are promoted with repeated oral administration of dextran sodium sulfate (DSS) to induce colonic inflammation [12]. Using this model, we examined the requirement of nuclear APC in suppression of colitis-associated CRC. In contrast to the small intestinal tumors that predominantly develop in Apc^{Min} mice, the AOM-DSS-treated mice develop tumors in the colon, the site of tumor formation in humans with mutated *APC* [13]. Furthermore, most mouse models with germline *Apc* mutations express truncated Apc lacking both β-catenin degradation and nuclear localization domains. However, *Apc* mutations are not typically found in colon tumors from AOM-DSS-treated mice. Rather, mutations in β-catenin that render it incapable of Apc-mediated destruction are considered the

initiating step [12]. This feature allows us to distinguish tumor suppressor functions of nuclear Apc from the cytoplasmic role of Apc in targeting β-catenin for destruction.

Here we show that nuclear Apc suppresses colon tumorigenesis in the AOM-DSS mouse model. We provide evidence that this tumor suppression is at the initiation stage of tumorigenesis. Based on the cumulative data obtained using the Apc^{mNLS} model, we propose that nuclear Apc protects against colitis-associated colon tumorigenesis by suppressing inflammation, inhibiting Wnt signaling and increasing colonocyte proliferation.

Materials and methods

Mouse Husbandry

Mice were maintained at the Animal Care Unit at the University of Kansas according to animal use statement number 137-01. The research complied with all relevant federal guidelines and institutional policies. Mice were fed ad libitum with Purina Lab Diet 5001 and were housed in cages in adjoining animal rooms. All mice are C57BL/6J and $Apc^{mNLS/mNLS}$ mice are congenic (>F15).

Analysis of mRNA from colon epithelial cells by real-time reverse transcription–PCR

Colon epithelial cells from $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ were isolated, mRNA was extracted and quantitative polymerase chain reaction (QPCR) of the cDNA was performed as described using primers listed in Table 4.1 [11].

Analysis of colons

Fifteen $Apc^{mNLS/mNLS}$ and 8 $Apc^{+/+}$ mice received an intra-peritoneal injection of 7.5 mg/Kg AOM at the age of 6 weeks followed by 3 cycles, 5 days each, of 2.5% of Dextran Sodium Sulfate (DSS) in the drinking water at 7, 10 and 13 weeks. Fourteen $Apc^{mNLS/mNLS}$ and 10 $Apc^{+/+}$ mice were not treated and served as control groups. Mice were sacrificed at 24 weeks, then the large intestine was removed, measured, opened longitudinally and fixed in 10% buffered

formalin overnight followed by 70% ethanol until use. Colonic tumors and lymphoid follicles were identified and measured by an investigator blind to the animal's genotype using a dissecting microscope with 10X magnification and with the aid of an eyepiece graticule calibrated to a 50-mm scale stage micrometer with 0.1 and 0.01-mm graduation (Leica).

Histopathological examination

All tumors and representative lymphoid follicles were dissected from the surrounding tissues, processed and paraffin-embedded. Sections (8 μm) were stained with hematoxylin-eosin and examined by a pathologist.

Detection of β-catenin and Kras mutations

DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen) from 3-8 manually microdissected, 8 μm sections of paraffin-embedded tissue for each tumor and amplified using primers spanning β-catenin exon 3 and Kras exon 1 listed in Table 4.2. Gel purified PCR products were sequenced (ACGT Inc.).

Assessment of proliferation

Paraffin embedded tumors were sectioned at 8-μm and stained for proliferation marker Ki-67 as described [11]. Images from at least three fields per tumor (100 μm x 100 μm) were analyzed for Ki-67 positive cells at 40X magnification.

Statistical Analysis

p-values were calculated using Student t-test, Mann-Whitney non parametric tests, Fisher's exact test and *GraphPad Prism* software as indicated in figure legends.

Table 4.1: Primers to quantify targets that were differentially expressed in $Apc^{Min/+}$ polyps

Gene	Forward primer	Reverse primer
Cox-1	5'-AAGGAGTCTCTCGCTCTGG-3'	5'-CTGGTTCTGGCACGGATAGT-3'

Cox-2	5'-CATTCTTTGCCCAGCACTTC-3'	5'-GGCGCAGTTTATGTTGTCTG-3'
CXCR-2	5'-AGCAGAGGATGGCCTAGTCA-3'	5'-TCCACCTACTCCCATTCCTG-3'
$P^{21\ Cip}$	5'-TTGCACTCTGGTGTCTGAGC-3'	5'-GGGCACTTCAGGGTTTTCTC-3'
MIP-2	5'-CAGACTCCAGCCACACTTCA-3'	5'-CAGTTCACTGGCCACAACAG-3'
HGPRT	5'- TGCTCGAGATGTCATGAAGG-3'	5'- TATGTCCCCCGTTGACTGAT-3'

Table 4.2: Primers for mutation analysis of β-catenin and K-Ras in tumors

β-catenin	5'-TTCAGGTAGCATTTTCAGTTCA-3'	5'-TGCTAGCTTCCAAACACAAATGC-3'
Kras	5'-TGTAAGGCCTGCTGAAAATG-3'	5'-GCACGCAGACTGTAGAGCAG-3'

Results

Increased expression of inflammatory mediators in colon epithelial cells from $Apc^{mNLS/mNLS}$ mice

Loss of the wildtype *Apc* allele in intestinal epithelial cells of Apc^{Min} mice resulted in development of tumors that showed differential gene expression. Although some up-regulated genes were not previously characterized Wnt targets, many were, illustrating the importance of Apc in antagonizing Wnt signal-induced cellular proliferation [14]. Our lab previously showed that $Apc^{mNLS/mNLS}$ mice have higher expression of Wnt target genes in intestinal epithelial cells [11]. To determine if other genes reported to be differentially expressed in apparently normal colon epithelial cells of Apc^{Min} mice also have altered expression in $Apc^{mNLS/mNLS}$ mice, expression levels of Cyclo-oxygenase-1 (*Cox-1*), Cyclo-oxygenase-2 (*Cox-2*), *CXCR-2*, $p21^{Cip}$ and Macrophage inflammatory protein-2 (*MIP-2*) were compared in colon epithelial cells from $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ mice. There were no significant differences in the expression levels of *Cox-1*, *CXCR-2* and $p21^{Cip}$ in $Apc^{mNLS/mNLS}$ samples relative to $Apc^{+/+}$ samples (data not shown).

However, *Cox-2* and *MIP-2* mRNA levels were each significantly higher in colon epithelial cells from $Apc^{mNLS/mNLS}$ compared with $Apc^{+/+}$ mice (Figure 4.1).

$Apc^{mNLS/mNLS}$ mice have higher susceptibility to colitis-associated colon tumorigenesis.

Inflammation is a major risk factor for the development of colorectal cancer [1]. Both Cox-2 and MIP-2 are involved in inflammation. Cox-2 is the rate-limiting step in arachidonic acid conversion to inflammatory mediators including prostaglandins [15]. MIP-2 is a pro-inflammatory chemokine [16]. The finding that both *Cox-2* and *MIP-2* are up-regulated in colon epithelial cells from $Apc^{mNLS/mNLS}$ mice indicates a potential role for nuclear Apc in inflammation. We previously showed that the Apc^{mNLS} allele increases intestinal tumorigenecity in mice harboring a germline mutation that results in Apc truncation, Apc^{Min} [11]. Considering these findings, we hypothesized a role for nuclear Apc in the protection against colitis-associated tumorigenicity. To test this hypothesis, we treated 6 week old $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ mice with a single dose of the mutagen Azoxymethane (AOM). This was followed by 3 cycles, 5 days each, of 2.5% Dextran Sodium Sulfate (DSS) administered in the drinking water at 7, 10 and 13 weeks (Figure 4.2A). Five of fifteen treated $Apc^{mNLS/mNLS}$ mice (33%) developed rectal prolapse and were sacrificed at age 20.6, 20.6, 21, 21 and 22.6 weeks, whereas only one of eight treated $Apc^{+/+}$ mice required early termination (at 22.9 weeks) due to rectal bleeding. The remaining mice were sacrificed at 24 weeks. In addition, two untreated control groups of $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ mice were sacrificed at 24 weeks of age, at which time none displayed detectable colon tumors. While all of the $Apc^{mNLS/mNLS}$ treated mice developed at least one colonic tumor, over one-third of the treated $Apc^{+/+}$ mice remained colon tumor-free at the end of the study (Figure 4.2B, 4.2C, 4.2D). Moreover, tumor numbers in treated $Apc^{mNLS/mNLS}$ mice were more than double that of treated $Apc^{+/+}$ mice (Figure 4.2E). Histopathological examination of 20 tumors from $Apc^{mNLS/mNLS}$ mice and 10 tumors from $Apc^{+/+}$ mice indicated that all were polypoidal,

semi-flat or flat adenomas with some degree of atypia (Figure 4.2F). Moderate to marked lymphoplasmacytic infiltration was observed in most tumors with varying degree of inflammatory reactions. We conclude that AOM-DSS-treated $Apc^{mNLS/mNLS}$ mice have higher tumor incidence and multiplicity than $Apc^{+/+}$ mice and that tumor histology is similar in all mice.

Nuclear Apc suppresses the initiation of colitis-associated tumorigenesis

To determine the mechanism by which Apc^{mNLS} increases tumor formation in the AOM-DSS model, we investigated several predicted contributory elements. Weight loss and shortening of colon length are used as inflammation parameters in AOM-DSS mouse models [17]. We found that treated $Apc^{mNLS/mNLS}$ mice lost significantly more weight than treated $Apc^{+/+}$ mice relative to the corresponding untreated control groups (Figure 4.3A). Colon lengths were reduced by 11% in treated $Apc^{mNLS/mNLS}$ mice and by only 8% in treated $Apc^{+/+}$ mice, but this trend did not reach statistical significance (Figure 4.3B). Untreated $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ mice had similar numbers of lymphoid follicles visible with the aid of a dissecting microscope and 10X magnification (Figure 4.3C). However, the treated $Apc^{mNLS/mNLS}$ mice had significantly more visible lymphoid follicles than the treated $Apc^{+/+}$ mice (Figure 4.3C). Together, these data suggest that there is more severe inflammation in AOM-DSS-treated $Apc^{mNLS/mNLS}$ mice than in treated $Apc^{+/+}$ mice.

Tumor size in treated $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ mice did not significantly differ (Figure 4.3D), nor did proliferation level as assessed by proliferation marker, Ki-67 (Figure 4.3E & F). Taken together, it appears that tumor growth is not affected in $Apc^{mNLS/mNLS}$ mice.

Tumors from treated $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ mice have similar spectrum of β-catenin mutations and no Kras mutations

Stabilizing mutations in β-catenin exon 3 and activating mutation of *Kras* codon 12 are characteristics of tumors from mice treated with AOM-DSS. We found mutations in β-catenin

exon 3 in each of the 10 $Apc^{+/+}$ tumors examined and in 24/25 $Apc^{mNLS/mNLS}$ tumors examined (Figure 4.4A). Furthermore, the specific missense mutations in β-catenin were similar in both groups (Figure 4.4B, 4.4C). No mutations were found in exon 1 of *kras* in tumors from either group (data not shown). These results implicate nuclear Apc in inhibiting initiation of inflammation-induced tumorigenicity in mice.

Discussion

Promiscuous Wnt signaling is a hallmark of CRC in both sporadic and colitis-associated cases but this promiscuity is the result of different genetic events. While *APC* mutation is the initiating event in most sporadic cases, APC-independent β-catenin activation is a typical early event in colitis-associated CRC. Interestingly, in colitis-associated-CRC, mutation of *APC* still occurs, however at a later stage of tumor formation [1]. This suggests two distinct tumor suppressor roles for APC that must be eliminated during CRC progression. Some APC function(s) need to be abolished early, including regulation of Wnt signaling, while the other activities must be eliminated later for the cancer to progress. In sporadic cases of colon cancer, both roles for APC are eliminated early by mutating *APC*. On the other hand, in colitis-associated cancer, the early development of a colon tumor is typically associated with APC-independent β-catenin nuclear translocation [1]. In a mouse model of colitis-associated CRC, colon tumors are initiated by a single injection of the mutagen AOM which leads to "stabilizing" mutations in β-catenin, followed by treatment with DSS to induce inflammation in the colon [12]. Injection with a single dose of AOM without subsequent DSS treatment results in only a very low tumor incidence [18], emphasizing that aberrant regulation of Wnt signaling by β-catenin stabilization is not sufficient for tumor formation. In humans, most colon cancers display mutations in *APC* while tumors in other organs often show mutations in other Wnt pathway components [4]. This difference could be explained, at least in part, if the second tumor

suppressor function of APC is particularly critical in the colon. In addition, nonsense mutations that truncate the C-terminal 200- 300 amino acids of APC do not alter Wnt signal regulation but are associated with increased tumorigenesis in both humans [19, 20] and in the AOM-DSS rat model [21]. In contrast, more severe truncation of APC can initiate tumor formation in both humans and rodent models, highlighting the importance of other tumor suppressor function(s) of APC. Here we provide evidence that nuclear APC functions in suppressing initiation of colitis-associated CRC.

In this study, we found that $Apc^{mNLS/mNLS}$ mice have increased colitis-associated tumor susceptibility as indicated by an increase in both tumor incidence and multiplicity after treatment with AOM-DSS. One possible explanation for this finding is that nuclear Apc suppresses inflammation in the colon. In support of this idea, we found up-regulation of the inflammatory mediators, Cox-2 and MIP-2 in normal colon epithelial cells from $Apc^{mNLS/mNLS}$ mice (Figure 4.1). Many inflammatory mediators are up-regulated in $Apc^{Min/+}$ polyps including Cox-1, Cox-2, MIP-2, osteopontin (OPN), CXCR-2, growth-related oncogene- α (Gro-α) [14]. While Cox-2 is generally considered a Wnt target [22-24], the other genes are not known to be regulated by Wnt signaling, suggesting a Wnt-independent role for Apc in gene regulation and protection against inflammation. In this study, colons from AOM-DSS-treated $Apc^{mNLS/mNLS}$ mice had more visible lymphoid follicles than those from treated $Apc^{+/+}$ mice. Lymphoid follicle incidence has been correlated with the degree of colonic inflammation and mucosal destruction [25]. Furthermore, tumors adjacent to lymphoid follicles have more cellular proliferation than tumors more distant from lymphoid follicles [25].

We found no change in tumor size or tumor cell proliferation in treated $Apc^{mNLS/mNLS}$ mice, suggesting nuclear Apc suppresses tumor initiation rather than tumor progression. In addition, there were no histo-pathological differences in tumors from $Apc^{+/+}$ and $Apc^{mNLS/mNLS}$

mice and they possessed the same spectrum of exon 3 β-catenin mutations. Together, these findings suggest loss of nuclear APC function initiates colon tumors at a step prior to Wnt signal dysregulation. The idea that *APC* mutation can initiate tumors before Wnt dysregulation is supported by findings in humans and in rodent and zebrafish models. In most early lesions from FAP patients, both copies of APC are mutated, however aberrant Wnt activation is not observed until lesions are advanced [26, 27]. In PIRC rats, which carry a germline mutation in Apc, nuclear β-catenin is detected in late but not early adenomas [28]. Furthermore, results from a study using a zebrafish model led to the proposal that Wnt signaling dysregulation is the second of two steps necessary for tumor initiation after *apc* mutation and the transcriptional co-repressor CtBP1 is required for the first step [29]. Of note, previous data had implicated nuclear APC in facilitating the nuclear import of CtBP1 [30].

Nuclear APC can repress Wnt target gene expression both *in-vivo* and in tissue culture cells. The proposed mechanisms for this repression, supported by analysis of the $Apc^{mNLS/mNLS}$ mouse model include; 1) nuclear APC sequestration of β-catenin to prevent interaction with LEF-1, and 2) APC facilitated interaction of the transcription repressor CtBP1 with the promoters of Wnt target genes [6, 11]. Considering these findings, higher Wnt target expression might contribute to the increased colitis-associated tumorigenicity in $Apc^{mNLS/mNLS}$ mice. We propose that in $Apc^{+/+}$ mice, nuclear Apc can reduce expression of Wnt target genes in cells with stabilized β-catenin by sequestering β-catenin from LEF-1 or by importing the co-repressor CtBP1. In contrast, in colon cells from $Apc^{mNLS/mNLS}$ mice, Apc nuclear import is compromised thus, Apc cannot repress transcription mediated by a stabilized β-catenin.

Inflammation and Wnt signal up-regulation are not completely independent factors. Inflammation can activate Wnt signaling through many pathways including NF-κB [1]. Moreover, it has been reported that *APC* mutations can alter retinoic acid metabolism, leading to

Wnt-independent up-regulation of Cox-2. The induced Cox-2 increases PGE_2 synthesis, which in turn activates Wnt signaling [31, 32]. On the other hand, Wnt signaling can increase inflammatory response through up-regulation of some inflammatory mediators including Nitric oxide synthase2 (NOS-2) [33] and Cox-2 [24]. Cox-2 is involved in colorectal tumor formation and often up-regulated in colon cancer [32]. Cox-2 inhibitors have been used to treat CRC [2, 34]. Besides being the rate limiting enzyme in the synthesis of inflammatory mediators, Cox-2 also induces the anti-apoptotic Bcl2 [35], the angiogenic VEGF [36]and the pro-tumor cytokine, IL-23 and inhibits the tumor suppressor cytokine IL-12 [37].

In conclusion, using the novel $Apc^{mNLS/mNLS}$ mouse model, we provide evidence that nuclear Apc contributes to suppression of colitis-associated CRC initiation. Further studies using the $Apc^{mNLS/mNLS}$ mouse model will allow elucidation of less characterized tumor suppressor functions of APC. Ultimately, this knowledge is expected to lead to better diagnostic, prognostic and therapeutic approaches for colorectal cancer.

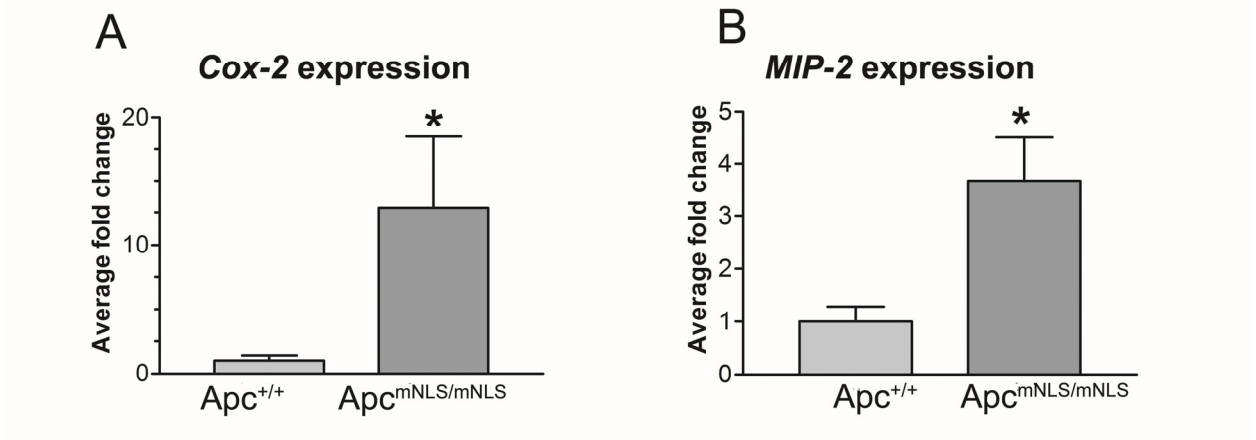
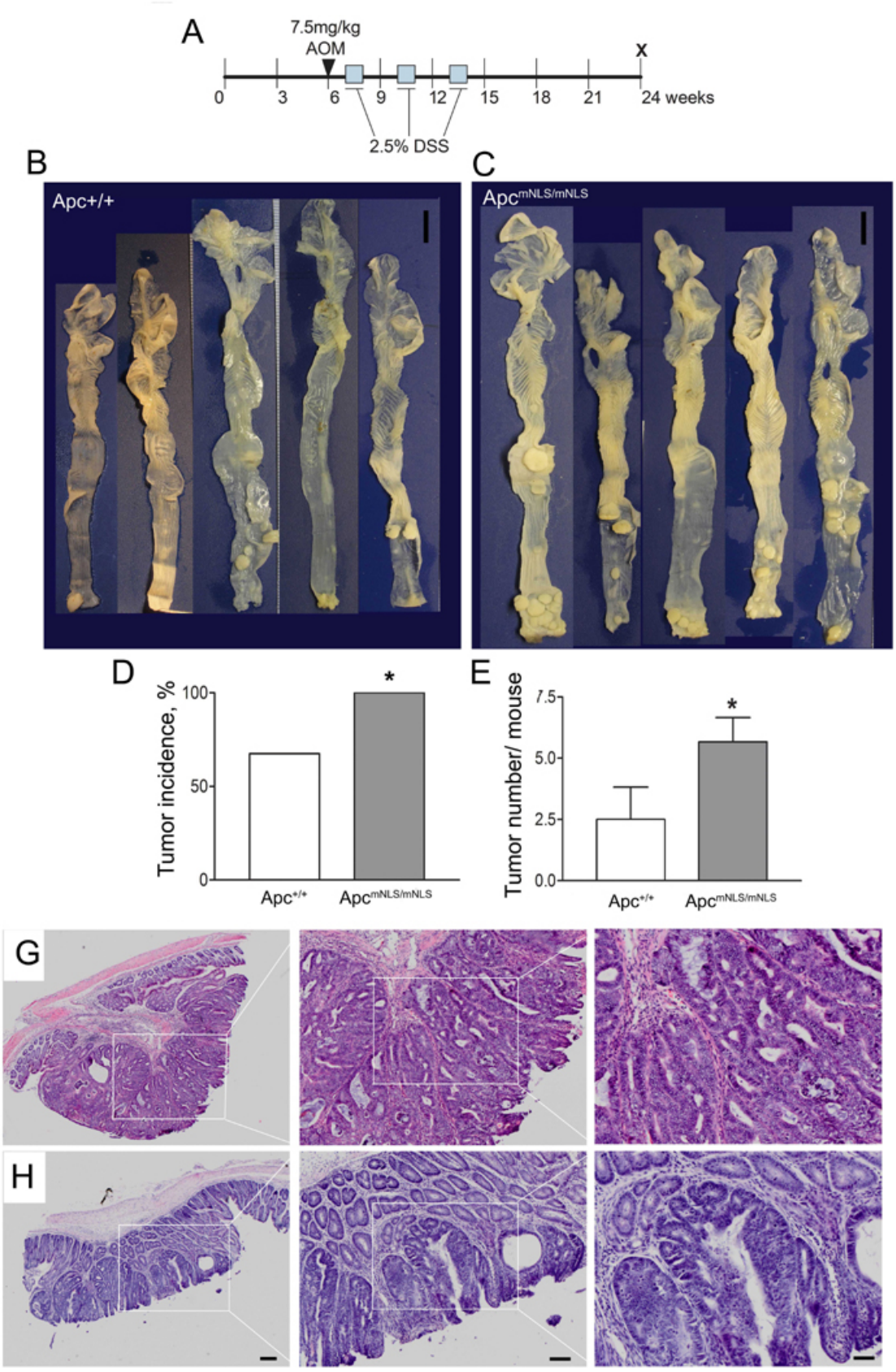


Figure 4.1: Differential expression of inflammatory mediators in Apc$^{mNLS/mNLS}$ mice
Cox-2 and *MIP-2* mRNA levels were each significantly higher in colon epithelial cells from Apc$^{mNLS/mNLS}$ mice than from Apc$^{+/+}$ mice ($p < 0.001$ for *Cox-2* and $p < 0.05$ for *MIP-2,* Mann-Whitney non-parametric test). Messenger RNA levels were normalized to mRNA for the housekeeping gene *HGPRT* and are presented as average fold change calculated using $\Delta\Delta C(t)$. Error bars represent s.e.m. "*" indicates statistical significance.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Tumor incidence, %
100
50
0
*
Apc+/+
ApcmNLS/mNLS
E
Tumor number/ mouse
7.5
5.0
2.5
0.0
*
Apc+/+
ApcmNLS/mNLS
G
H

Figure 4.2: Apc^{mNLS} allele increases tumor incidence and multiplicity in AOM-DSS mouse model. **(A)** AOM-DSS treatment protocol. Representative samples of large intestines from treated $Apc^{+/+}$ (n=8) **(B)** and $Apc^{mNLS/mNLS}$ (n=15) mice (**C**), scale bars = 1cm. **(D)** Tumor incidence was significantly higher in treated $Apc^{mNLS/mNLS}$ mice compared to treated $Apc^{+/+}$ mice ($p < 0.05$, Fisher's exact test). **(E)** $Apc^{mNLS/mNLS}$ mice had significantly more colon tumors than $Apc^{+/+}$ mice ($p < 0.05$, using student t-test). Error bars represent s.e.m and significant values are indicated with ***. (F)** Histopathology of representative polypoidal (top) and semi-flat (bottom) colonic tumors from treated $Apc^{mNLS/mNLS}$ mice. Higher magnifications of boxed areas are presented. Scale bars 200 μm (left panels), 100 μm (middle) and 50 μm (right).

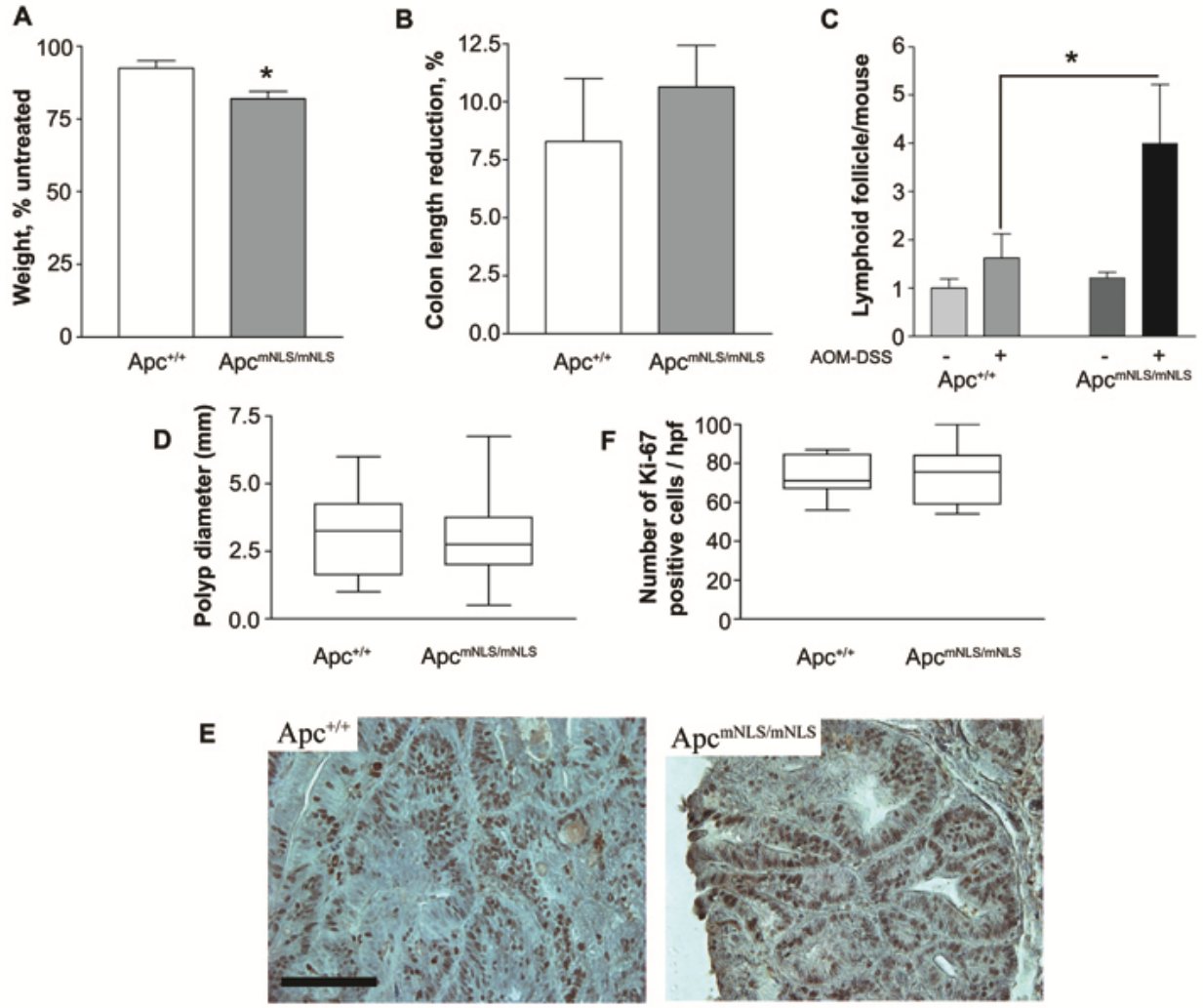

Figure 4.3: Nuclear Apc protects against colitis-associated tumor initiation rather than progression. (A) The average weight change of AOM-DSS-treated mice is shown relative to the untreated mice from both $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ groups. $Apc^{mNLS/mNLS}$ mice lost significantly more weight than $Apc^{+/+}$ mice ($p<0.01$, using Student t-test). **(B)** The reduction of colon length in treated $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ mice relative to their respective untreated control groups is presented as average ± s.e.m. ($p=0.24$) **(C)** The average number of visible lymphoid follicles was significantly more in treated $Apc^{mNLS/mNLS}$ mice relative to treated $Apc^{+/+}$ mice ($p<0.05$, using Student t-test) (**D)** The diameters of colon tumors from treated $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ mice presented as box and whiskers plots showed no significant difference in the size of the tumors from each treated mouse group ($p>0.05$, using Student t-test). **(E)** Box and whiskers plots of proliferation marker Ki-67-positive cells per (100 μm x 100 μm) high-power field (hpf) from $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ mice showed no significant difference between groups ($p>0.05$, using Student t-test). **(F)** Representative images of Ki-67 staining, scale bar = 100 μm.

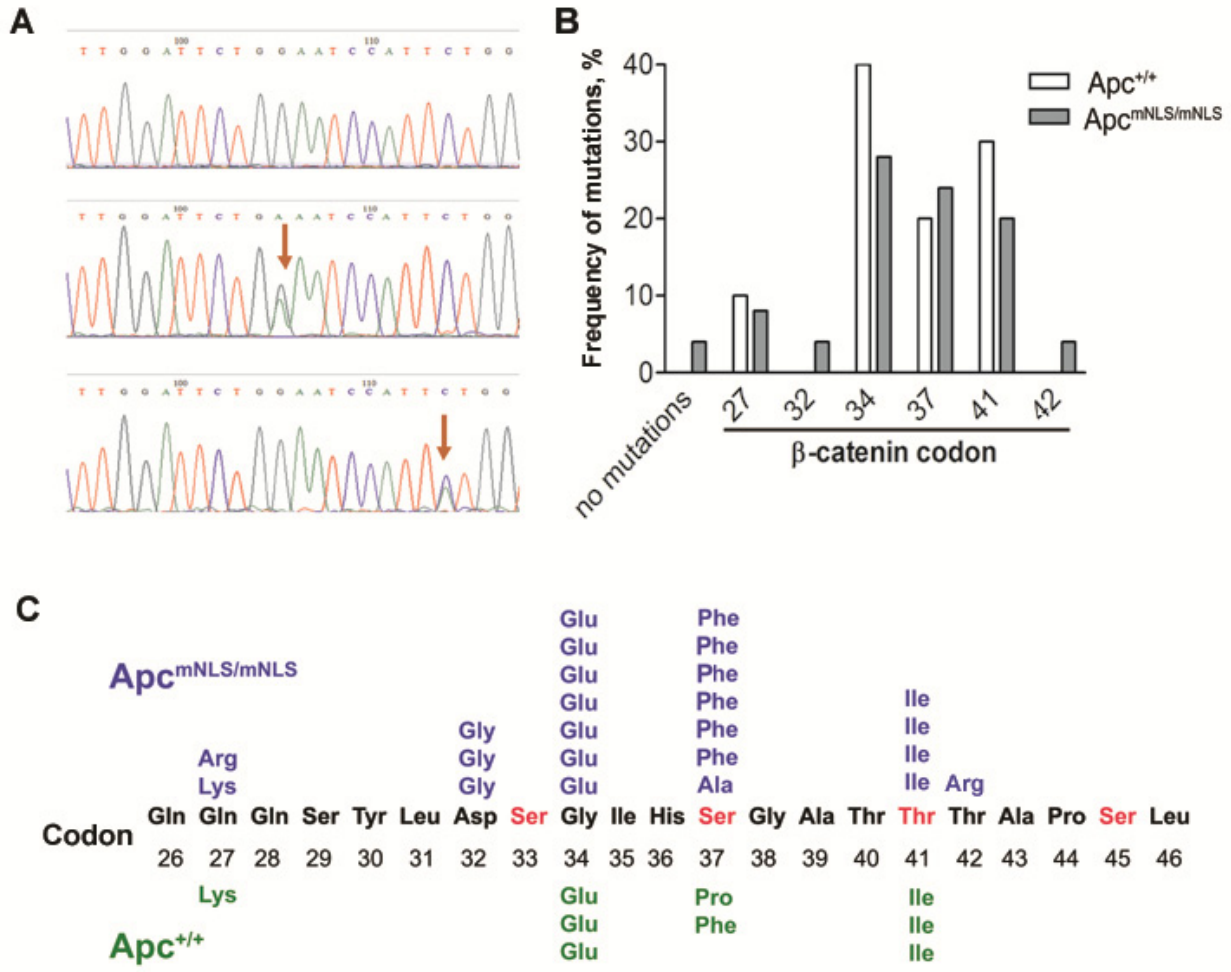

Figure 4.4: Tumors from treated Apc$^{mNLS/mNLS}$ and Apc$^{+/+}$ mice have the same spectrum of β-catenin mutations. (A) Representative sequencing chromatographs showing a G->A (middle) or a C->T (lower) mutation (arrows) or the normal control β-catenin coding sequence (top). **(B)** The distribution of β-catenin exon 3 mutations found in tumors from Apc$^{mNLS/mNLS}$ and Apc$^{+/+}$ mice is displayed as the relative frequency at each codon. **(C)** The predicted amino acid alterations resulting from the missense mutations in exon 3 of β-catenin are presented for tumors from treated Apc$^{mNLS/mNLS}$ (top) and Apc$^{+/+}$ (bottom) mice.

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Chapter 5

Loss of heterozygozity and extra-intestinal phenotypes of $Apc^{mNLS/NLS}$ mice

Abstract

This chapter contains additional data from studies of the $Apc^{mNLS/Min}$ mice. The chapter is divided into two parts. In the first part, we show that polyps from $Apc^{mNLS/Min}$ mice show loss of heterozygosity (LOH) with loss of the Apc^{mNLS} allele. We discuss possible explanations and our future directions. In the second part, we show that $Apc^{mNLS/Min}$ mice have more severe anemia, enlarged spleens and enhanced mammary tumorigenicity relative to $Apc^{Min/+}$ mice, which indicates that Apc^{mNLS} enhances extra-intestinal phenotypes in Apc^{Min} mice. We also provide data regarding LOH in two mammary tumors that developed in $Apc^{mNLS/Min}$ mice; loss of Apc^{Min} appears to occur in one tumor

Part 1: Loss of heterozygosity (LOH) in intestinal polyps from $Apc^{mNLS/Min}$ mice

Introduction

As described in Chapter 2, there is loss of the wild-type *Apc* allele in most intestinal tumors that develop in $Apc^{Min/+}$ mice [1, 2]. This loss of heterozygosity (LOH) is thought to be required for tumor formation in these mice. $Apc^{mNLS/mNLS}$ mice typically do not develop intestinal polyps. However, as shown in Chapter 3, Apc^{Min} mice that carry the Apc^{mNLS} allele ($Apc^{mNLS/Min}$) develop more intestinal tumors, with tumors larger in size than $Apc^{Min/+}$ mice [3]. To test for LOH in intestinal polyps from $Apc^{mNLS/Min}$ mice, we measured the relative abundance of the Apc^{mNLS} and Apc^{Min} alleles in genomic DNA extracted from mouse tails and intestinal polyps using the quantitative real-time polymerase chain reaction (qPCR). We used 2 TaqMan® probes, each labeled with a

different fluorescent dye (6-FAM and HEX), and specific for the *Apc*^{mNLS} or *Apc*^{Min} allele, respectively. We found a reduction in relative abundance of the *Apc*^{mNLS} allele and an increase in relative abundance of the *Apc*^{Min} allele in genomic DNA isolated from intestinal polyps compared to DNA from tails of Apc$^{mNLS/Min}$ mice. We concluded that LOH occurs in polyps from Apc$^{mNLS/Min}$ mice, resulting in loss of the *Apc*^{mNLS} allele.

Materials and methods

Genomic DNA extraction

For each of 5 Apc$^{mNLS/Min}$ mice, three ileal tumors were isolated from freshly dissected, longitudinally opened, small intestines with the aid of a dissecting microscope. The tumors were digested using 100 µg proteinase K in a 200-µl total reaction volume at 55^{o} C for 2 hours, followed by inactivation of the enzyme by heating at 95 ^{o} C for 5 minutes. Genomic DNA from the tumors and tails was then purified using the standard phenol/chloroform separation method and DNA from 3 tumors from each mouse was combined for analysis.

Quantitative PCR

Primers were designed to flank Apc-NLS2 and produce a PCR product of 152 bp. Two TaqMan® probes were designed to recognize either mutant Apc-NLS2 sequence (*Apc*^{mNLS} allele) or the wild-type sequence (found in the *Apc*^{Min} allele); table 5. 1. The mutant NLS2 probe has 6-FAM and the wild-type probe has HEX at its 5' end, and both probes include the quencher Iowa black® IDT (IABkFQ). PCR reactions were carried out using an Opticon 2 DNA Engine Thermocycler with every reaction performed in duplicate. The reaction mix contained 1X HS DyNamo qPCR mix (NEB), 15 picomoles of each primer and probe, and approximately 250 ng DNA. The reaction was initially

heated at 95 ^{o} C for 15 minutes to activate the polymerase followed by 40 cycles of denaturation at 94 ^{o} C for 20 seconds, annealing at 54 ^{o} C for 20 seconds, and extension at 72 ^{o} C for 30 seconds. This was followed by detection of fluorescence. The change of the relative abundance of the *Apc*^{mNLS} to *Apc*^{Min} alleles in the tumor and tail DNA from every mouse was calculated using ΔC(t).

Table 5. 1: primers used in qPCR for LOH analysis in polyps from Apc$^{mNLS/Min}$ mice

Primer (probe)	**Sequence**
NLS2 QPCRF	5'-TTC TAG GGC TCT GCT TTT CG-3'
NLS2 QPCRR	5'-CGA AGA CAC CCC TGT CTG TT-3'
NLS2 TaqMan probe (*Apc*^{mNLS} probe)	5'-6-FAM/AGG CCT TTT TGC GGC C-3'IABkFQ
NLS2 TaqMan probeN (*Apc*^{Min} probe)	5'-HEX/CCA AAA AAG AAA AGG CCT TCA AG-3' IABkFQ

Results

We found a significant reduction in abundance of the *Apc*^{mNLS} allele compared to the *Apc*^{Min} allele in the polyps relative to the tail DNA of the same mice (the relative abundance is 0.05, $p =$ 0.015873, Mann-Whitney test); figure 5.1A.

Discussion

LOH of the wild-type *Apc* allele is required for the development of intestinal polyps in Apc$^{Min/+}$ mice [1, 2]. The mechanism of LOH in Apc$^{Min/+}$ mice is believed to be either complete loss and duplication of the whole chromosome carrying *Apc* (chromosome 18) or somatic recombination involving the q arm of the acrocentric chromosome 18. This LOH results in two copies of the mutant allele (*Apc*^{Min}) in polyp cells [4, 5]. We found that polyps from Apc$^{mNLS/Min}$ mice have an increased abundance of the *Apc*^{Min} allele relative to the *Apc*^{mNLS} allele, consistent with loss of *Apc*^{mNLS} allele in

these polyps (figure 5. 1A). Although our data suggest duplication of *Apc*^{Min} allele as well (we see increased amounts of the *Apc*^{Min} allele in the polyps), comparing the abundance of both alleles with another DNA locus on another chromosome will allow us in the future to differentiate between complete loss and "loss and duplication" of the *Apc*^{Min} allele in the polyps.

Our results of loss of the *Apc*^{mNLS} allele in polyps from Apc$^{mNLS/Min}$ mice are interesting in light of the data showing that Apc$^{mNLS/Min}$ mice develop more intestinal polyps than do Apc$^{Min/+}$ mice. Furthermore, the polyps from Apc$^{mNLS/Min}$ mice are larger and have a higher proliferative index compared to those from Apc$^{Min/+}$ mice[3]. Considering that the *Apc*^{mNLS} allele is lost in polyps from Apc$^{mNLS/Min}$ mice and the *Apc*$^{+}$ allele is lost in the polyps from Apc$^{Min/+}$ mice, why would there be an increase in the number (figure 5. 1B), size, and proliferative index of polyps from Apc$^{mNLS/Min}$ mice relative to Apc$^{Min/+}$ mice?

A non-autonomous role for the *Apc*^{mNLS} allele in intestinal tumorigenicity that increases the rate of tumor growth after the occurrence of LOH could provide one explanation. Enhanced tumor growth could increase the number of polyps detected, by allowing these polyps to reach the detection size limit earlier. Recently, it has been shown that a conditional mutation of *Apc* in uterine stroma results in development of endometrial tumors, a result that supports a non-autonomous role for *Apc* in other tissues [6, 7]. Alternatively, the *Apc*^{mNLS} allele may increase the number of polyps in Apc$^{mNLS/Min}$ mice by increasing the rate of LOH in these cells. Mutation of Apc has been associated with chromosomal instability [8]. Furthermore, nuclear APC interacts with TopoIIα, an enzyme important for DNA replication [9]. Another possible mechanism is through

activation of Wnt signaling in cells with the *Apc*^{mNLS} allele. Wnt signaling increases chromosomal instability [10, 11]. We have shown that the *Apc*^{mNLS} allele has a role in stimulating Wnt signaling activity in intestinal epithelial cells (Chapter 3) [3].

One caveat to consider when interpreting the experimental data is that the DNA from 3 different polyps from the ileum was combined for each mouse. It is possible that loss of the *Apc*^{mNLS} allele does not occur in every polyp. To test this possibility, we will extract DNA from individual polyps from jejunums, ileums and colons from different Apc$^{mNLS/Min}$ mice, and will test for LOH. Another important note is that we detected, albeit in very small amounts, the *Apc*^{mNLS} allele in the polyps. We think that this trace amount of *Apc*^{mNLS} allele represents the normal tissues in the polyps such as vascular and connective stromal tissue. To test this prediction, we are planning to stain polyps from these mice with an antibody that recognizes the c-terminal portion of Apc. This antibody should recognize the Apc^{mNLS} but not Apc^{Min} protein and will allow visualization of LOH in individual tumor cells within the polyps. In conclusion, our preliminary data suggest that loss of the *Apc*^{mNLS} allele occurs in intestinal tumors that develop in Apc$^{mNLS/Min}$ mice.

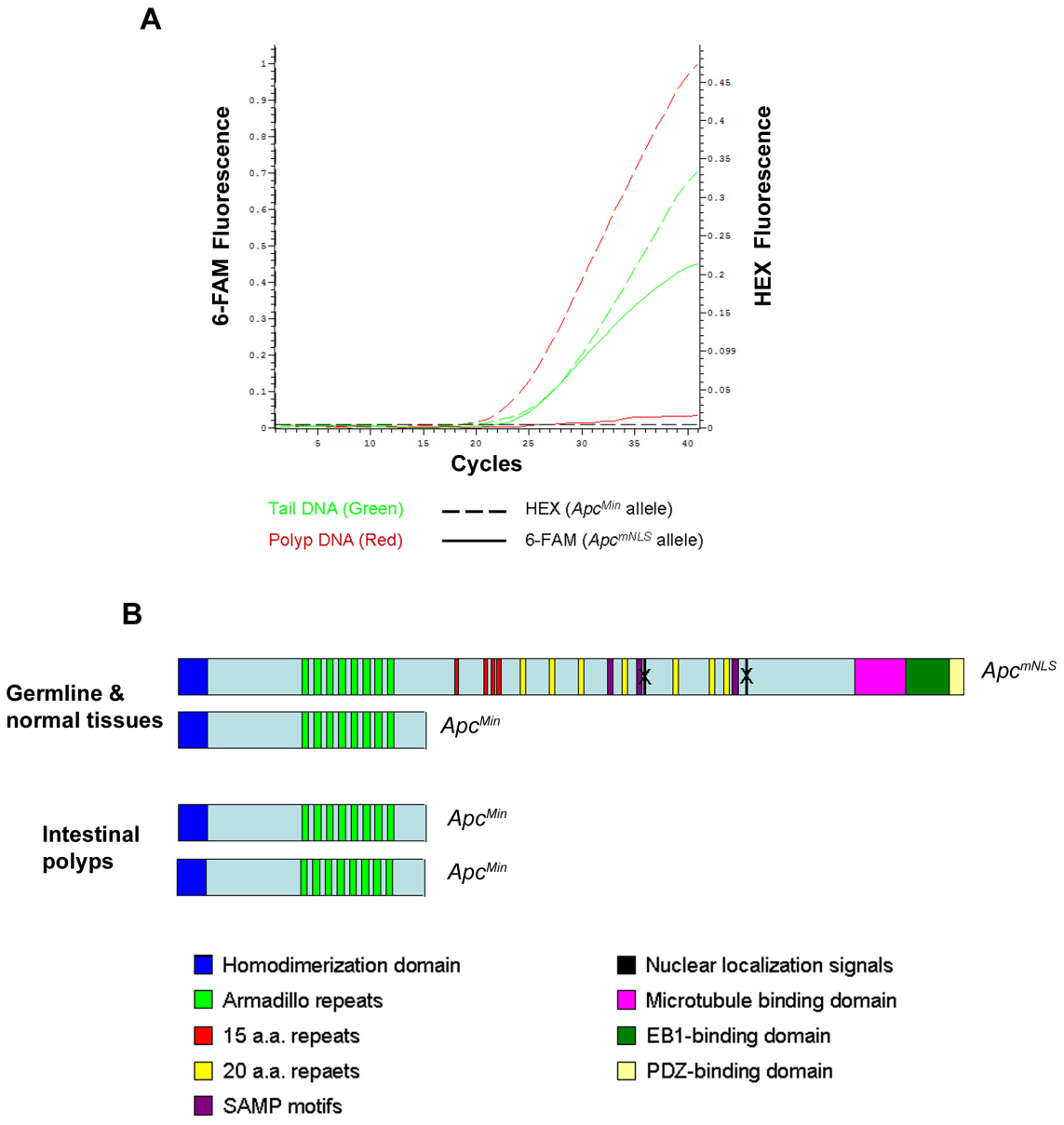

Figure 5.1: **LOH in intestinal polyps from Apc$^{mNLS/Min}$ mice**. **(A)** A representative example of real-time PCR results quantifying the *Apc^{mNLS}* (solid lines) and *Apc^{Min}* (dashed lines) alleles from genomic DNA extracted from three intestinal polyps (red) and the tail of the same mouse (green). There is a decrease in the abundance of the *Apc^{mNLS}* allele compared to that of the *Apc^{Min}* allele in intestinal polyps. **(B)** A diagram shows the Apc protein structure predicted in normal cells and in intestinal polyps.

Part 2: The *Apc*^{mNLS} allele enhances extra-intestinal phenotypes in Apc^{Min} mice

Introduction

In addition to intestinal polyps, Apc$^{Min/+}$ mice develop other manifestations outside the gastrointestinal tract [12]. Apc$^{Min/+}$ mice develop progressive microcytic-hypochromic anemia [13]. Chronic blood loss from intestinal polyps is thought to be the etiology of this anemia. Other studies, however, showed that changes in the bone marrow microenvironment of Apc$^{Min/+}$ mice may also participate in anemia development [14]. Apc$^{Min/+}$ mice also develop large spleens (splenomegaly) [14]. Another interesting extra-intestinal phenotype seen in older female Apc$^{Min/+}$ mice is the development of mammary tumors [15]. This mammary tumorigenesis is characterized by low penetrance (~5%) and late development (16 ± 3.5 weeks) [15]. Other extra-intestinal phenotypes in Apc$^{Min/+}$ mice include changes in immune cell production, gonadal abnormalities, and abnormal lipid profiles (see chapter 2) [14, 16-18].

Mice carrying both the *Apc*^{Min} and *Apc*^{mNLS} alleles showed enhanced intestinal tumorigenicity [3]. We examined Apc$^{mNLS/Min}$ mice for extra-intestinal manifestations and found these to be more severe as well. Apc$^{mNLS/Min}$ mice develop more severe anemia and larger spleens compared to Apc$^{Min/+}$ siblings. Two out of seven 17-weeks-old Apc$^{mNLS/Min}$ females developed mammary tumors while none of seven Apc$^{Min/+}$ female littermates developed mammary tumors. These data suggest that the *Apc*^{mNLS} allele also enhances extra-intestinal phenotypes in Apc^{Min} mice.

Materials and methods

Dissection and weighing of liver, spleens and mammary tumors

After being euthanized by use of CO_2, $Apc^{mNLS/Min}$, $Apc^{Min/+}$ and $Apc^{+/+}$ mice were weighed. The livers and spleens were removed, washed in PBS, weighed, and fixed in 10% saline-buffered formalin overnight, then soaked in 70% ethanol. The ratio of liver and spleen weight to total body weight per mouse was determined. Mammary tumors found in two older $Apc^{mNLS/Min}$ mice were dissected from the skin and processed in a similar manner.

Complete blood count analysis

Immediately after euthanasia with CO_2, blood (0.5 - 1 ml) was drawn from the mouse by means of intra-cardiac puncture with a 20G heparinized needle. The blood was sent to "Physician Reference Laboratory" (Overland Park, KS) for complete and differential blood count (CBC) analysis.

Genomic DNA extraction from mammary tumors

About 1 mg of fixed tumor tissue was isolated from the tumor by use of a new scalpel and then minced into small pieces. DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen) according to manufacturer instructions.

Determination of LOH in mammary tumors

Q-PCR for the relative abundance of the Apc^{mNLS} allele relative to the Apc^{Min} allele in genomic DNA from mammary tumors and tails of the same mice was performed as described previously for the polyp analysis. Samples were analyzed twice in duplicate.

Histopathological examination of mammary tumors

Wedge-shaped regions of each tumor extending from the outer surface to the center were paraffin-embedded. Sections (8 μm) were stained with hematoxylin-eosin

and examined by a murine pathologist, Dr. Ruth Sullivan at the University of Wisconsin Medical Center.

Results

$Apc^{mNLS/Min}$ mice have more severe hematological abnormalities than do $Apc^{Min/+}$ mice

Blood collected from 10 $Apc^{+/+}$, 21 $Apc^{Min/+}$ and 13 $Apc^{mNLS/Min}$ mice was analyzed for complete and differential blood count. There were no significant differences in platelet counts or in total or differential white blood cell counts (table 5. 2). However, $Apc^{mNLS/Min}$ mice developed significantly more severe anemia than did $Apc^{Min/+}$ or $Apc^{+/+}$ mice, as indicated by the reduction of hemoglobin (Hg) concentration, hematocrit (HCT) value, and red blood cell (RBC) count (figure 5. 2 A-C).

The average spleen weight as a percentage of total body weight is greater in $Apc^{mNLS/Min}$ mice (0.82%) than in $Apc^{+/+}$ (0.4%) and $Apc^{Min/+}$ (0.48%) mice. This trend, however, did not reach statistical significance (p= 0.054 and 0.09 respectively), as shown in figure 5. 2 D. There was not a significant difference in the normalized liver weight among different *Apc* genotypes (data not shown).

The Apc^{mNLS} allele enhances mammary tumorigenicity in Apc^{Min} mice

$Apc^{Min/+}$ female mice develop mammary tumors, although with a low penetrance (5%) and at relatively older ages [15]. We found that at 17 ± 1 weeks of age, 2 of 7 older $Apc^{mNLS/Min}$ females and 0 of 7 $Apc^{Min/+}$ littermates developed mammary tumors. The weights of the tumors were 3.2g (mouse #649) and 0.55g (mouse #650). Both tumors were firm in consistency, lobular, white in color, and attached to the skin but freely movable over the chest wall. When examined for histopathology (figure 5. 3 A-B), both

tumors were squamous, with nodules of extensive keratin production and pilar tumors. No signs of invasion were seen in the mammary tumor from mouse #649 while #650 showed extension of the tumor around skeletal muscle, suggestive of minimal invasion.

LOH in mammary tumors from $Apc^{mNLS/Min}$ mice

To gain insight as to the mechanism of mammary tumor development in $Apc^{mNLS/Min}$ mice, we measured the relative abundance of the Apc^{mNLS} and Apc^{Min} alleles in both mammary tumors and in tails from the same mice. In one mouse (#649) there was reduction, although small, of the proportion of the Apc^{mNLS} allele in the tumor tissues relative to tail tissue (figure 5. 3 C). Surprisingly, there was marked reduction of the Apc^{Min} allele in the tumor cells of the other mouse (#650), as shown in figure 5. 3 D.

Discussion

$Apc^{Min/+}$ mice develop intestinal and extra-intestinal phenotypes [19]. $Apc^{mNLS/Min}$ mice develop more severe intestinal polyposis than do $Apc^{Min/+}$ mice. Furthermore, the polyps that develop in $Apc^{mNLS/Min}$ mice are larger and have more mitotic cells, as indicated by positive staining for Ki-67 antigen [3]. We found that $Apc^{mNLS/Min}$ mice develop more severe extra-intestinal phenotypes than do $Apc^{Min/+}$ mice. $Apc^{mNLS/Min}$ mice have more severe anemia, larger spleens, and increased incidence of mammary tumors. Although these extra-intestinal manifestations do not include all of the extra-intestinal phenotypes described for $Apc^{Min/+}$ mice, they are consistent with more severe phenotypes in $Apc^{mNLS/Min}$ relative to $Apc^{Min/+}$ mice, which implies a role for nuclear Apc in extra-intestinal functions of Apc. Other described extra-intestinal phenotypes in Apc^{Min} mice, including abnormal lipid profile, abnormal testicular architecture, and aberrant immunocyte maturation and abundance, could also be examined in the future.

The underlying mechanism for the extra-intestinal phenotypes in $Apc^{Min/+}$ is not completely understood [14]. Anemia in $Apc^{Min/+}$ mice is thought to result from chronic blood loss in feces as the result of intestinal polyposis [13]. As $Apc^{mNLS/Min}$ mice develop more intestinal polyps than do $Apc^{Min/+}$ mice [3], the severe anemia seen in $Apc^{mNLS/Min}$ mice could represent this enhanced intestinal polyposis. However, studies have also shown haploinsufficiency for the Apc^{Min} allele in hematopoietic stem cell homeostasis in the bone marrow in $Apc^{Min/+}$ mice [16, 17]. Based on this observation, nuclear Apc may have a role in maintaining the bone marrow microenvironment. Future work could investigate this possibility.

$Apc^{Min/+}$ mice also develop larger spleens [12]. These spleens have extra-medullary hematopoiesis, suggesting a compensatory mechanism for anemia as their underlying cause [20]. However, You *et al.* have shown that enlarged spleens could develop in non-anemic $Apc^{Min/+}$ mice, which indicates that other factors may predispose these mice to enlarged spleens [14]. $Apc^{mNLS/Min}$ mice also develop larger spleens compared to $Apc^{Min/+}$ littermates. These large spleens could represent either a compensatory mechanism to more severe anemia in these mice or a role of nuclear Apc in the spleen. Pathological and functional analyses of these spleens could provide clues as to the mechanism of this phenotype. Interestingly, first generation $Apc^{mNLS/mNLS}$ mice (N1) showed enlarged spleens. Pathological analysis of some of these spleens from $Apc^{mNLS/mNLS}$ mice showed malignant transformation of immune cells causing histiocytic sarcoma (data not shown). This again supports a role for nuclear Apc in immunocyte homoeostasis.

Another tumor susceptibility phenotype that was observed in $Apc^{mNLS/Min}$ female mice is mammary tumorigenesis. Mammary tumors have been described in $Apc^{Min/+}$ mice, but at low incidence and at relatively older ages. In our colony, we found that 2/7 $Apc^{mNLS/Min}$ mice developed mammary tumors by age 17 weeks, while none of the 7 $Apc^{Min/+}$ littermates developed recognizable mammary tumors. At the histopathological level, the mammary tumors that developed in $Apc^{mNLS/Min}$ mice were indistinguishable from those described for $Apc^{Min/+}$ mice, consistent with a more severe phenotype rather than a completely different one. Another important note is that the number of mice in this experiment is relatively small. We hope to raise more old female mice of both genotypes for a more systematic analysis of mammary tumorigenicity. However, considering the low penetrance of the phenotype, the number of older female mice needed for such an experiment makes this experiment costly.

Analysis of LOH in mammary tumors from $Apc^{mNLS/Min}$ mice revealed loss of the Apc^{Min} allele in one tumor and loss of the Apc^{mNLS} allele in the other tumor. Interestingly, the tumor with signs of local invasion is the one that showed loss of the Apc^{Min} allele. This is interesting because we have not seen increased mammary tumor susceptibility in $Apc^{mNLS/mNLS}$ female mice. Although we do not have a complete explanation for this apparent paradox, some considerations should be taken into account. It is thought that mutation of *Apc* is not enough for the development of mammary tumors [21]. The reduction of the relative abundance of either allele was not as dramatic as seen in intestinal polyps. Although, this could represent the inclusion of normal stroma, it could also indicate that LOH happens in some, but not all cells. A laser-capture micro-

dissection of different regions of the tumors followed by staining of these tumors with antibodies against the Apc c-terminus may answer this question.

In conclusion, $Apc^{mNLS/Min}$ mice show enhanced extra-intestinal phenotypes including anemia, enlarged spleens, and enhanced mammary tumorigenesis. Future work will uncover the role for nuclear Apc in extra-intestinal tissues.

Table 5. 2: CBC with differential blood count in $Apc^{+/+}$, $Apc^{Min/+}$, and $Apc^{mNLS/Min}$ mice

	$Apc^{+/+}$ n= 10 Mean± SEM	**$Apc^{Min/+}$** n= 21 Mean± SEM	**$Apc^{mNLS/Min}$** n = 13 Mean± SEM
Red blood cell count (RBC) (x 10^6/mm^3)	7.93± 0.21	7.1± 0.24	5.99± 0.38*
Hemoglobin concentration (gm/dl)	12.56± 0.26	11.2± 0.37	9.61± 0.63*
Hematocrit (%)	38.01± 0.97	36.33± 1.24	30.33± 1.8*
Mean Corpuscular Volume (MCV) (fL)	48.02± 0.69	51.2 ± 0.56	50.88± 1.13
Mean Corpuscular Hemoglobin levels (MCH) (pg)	16.0± 0.22	15.78± 0.12	16.02± 0.24
Mean Corpuscular Hemoglobin Conc. (%)	33.38± 0.59	30.9± 0.40	31.68± 0.88
Platelet Count (x 10^3/ mm^3)	869± 70	913± 69	953± 102
White Blood Cell Count (WBC) (x 10^3 /mm^3)	10.64± 2.2	10.55± 0.77	9.77± 1.1
Differential WBC count			
Segmented neutrophils (%)	9.11± 1.97	9.11± 1.98	13.38± 2.84
Immature neutrophils (%)	0	0	0
Lymphocytes (%)	75.78± 8.8	75.78± 8.79	81.85± 3.47
Atypical lymphocytes (%)	0.56± 0.32	0.56± 0.32	1.62± 0.71
Monocytes (%)	2.17± 0.67	2.17± 0.67	2.54± 0.40
Eosinophils (%)	1.49± 0.43	1.49± 0.31	0.62± 0.21
Basophils (%)	0.31± 0.22	0	0
Metamylocytes (%)	0	0	0
Myelocytes (%)	0	0	0
Blast cells (%)	0	0	0
Nucleated RBC (%)	0	0.78± 0.44	0.5± 0.27
Abnormal RBC (polychromasia, anisocytosis, crenated RBCs, Howel Jolly)	Occasional	Occasional	Occasional

* $p< 0.05$

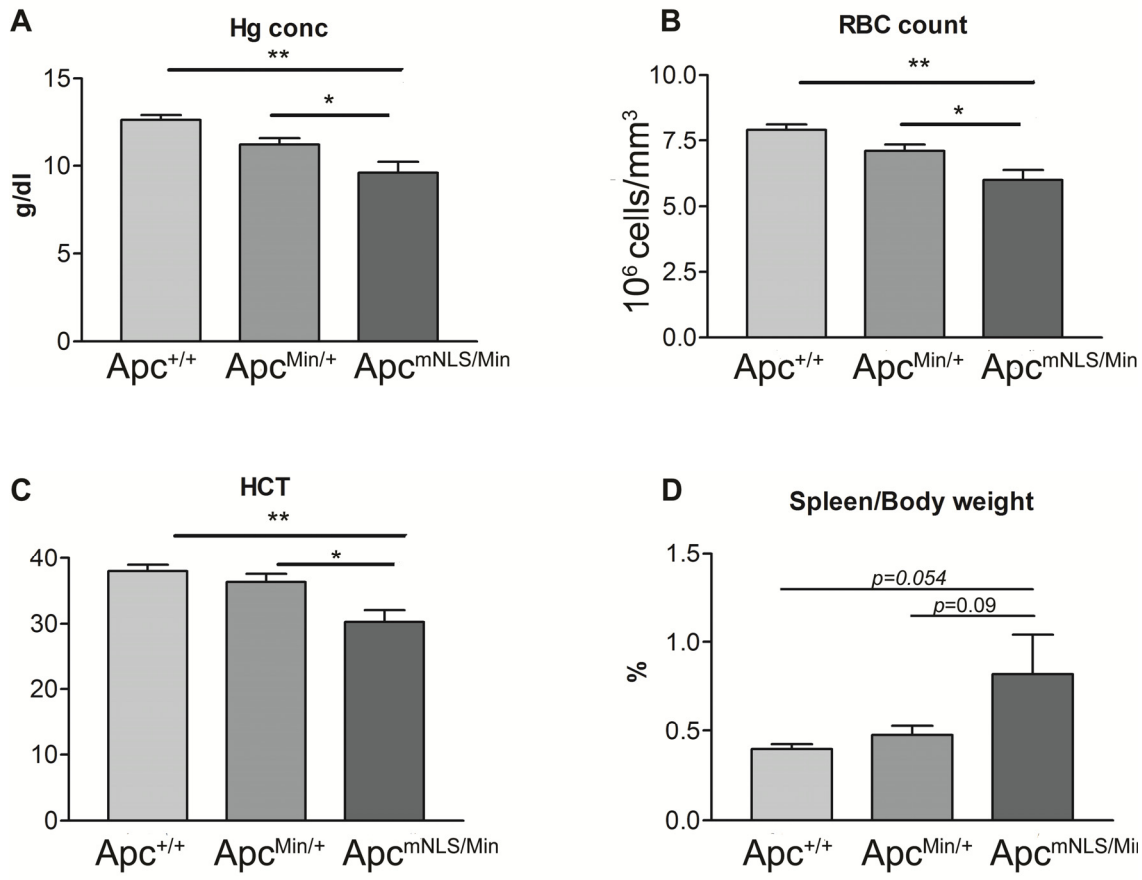

Figure 5. 2: Apc$^{mNLS/Min}$ mice develop more severe anemia and have larger spleens than do Apc^{Min} mice. (**A-C**) Complete blood count from 10 Apc$^{+/+}$, 21 Apc$^{Min/+}$ and 13 Apc$^{mNLS/Min}$ show that Apc$^{mNLS/Min}$ mice have significantly lower hemoglobin (Hg) concentration, red blood cell (RBC) count, and hematocrit (HCT) value relative to those of Apc$^{+/+}$ and Apc$^{Min/+}$ mice. **D** percentage of spleen weight relative to body weight in Apc$^{+/+}$, Apc$^{Min/+}$ and Apc$^{mNLS/Min}$ mice. Error bars represent SEM. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively.

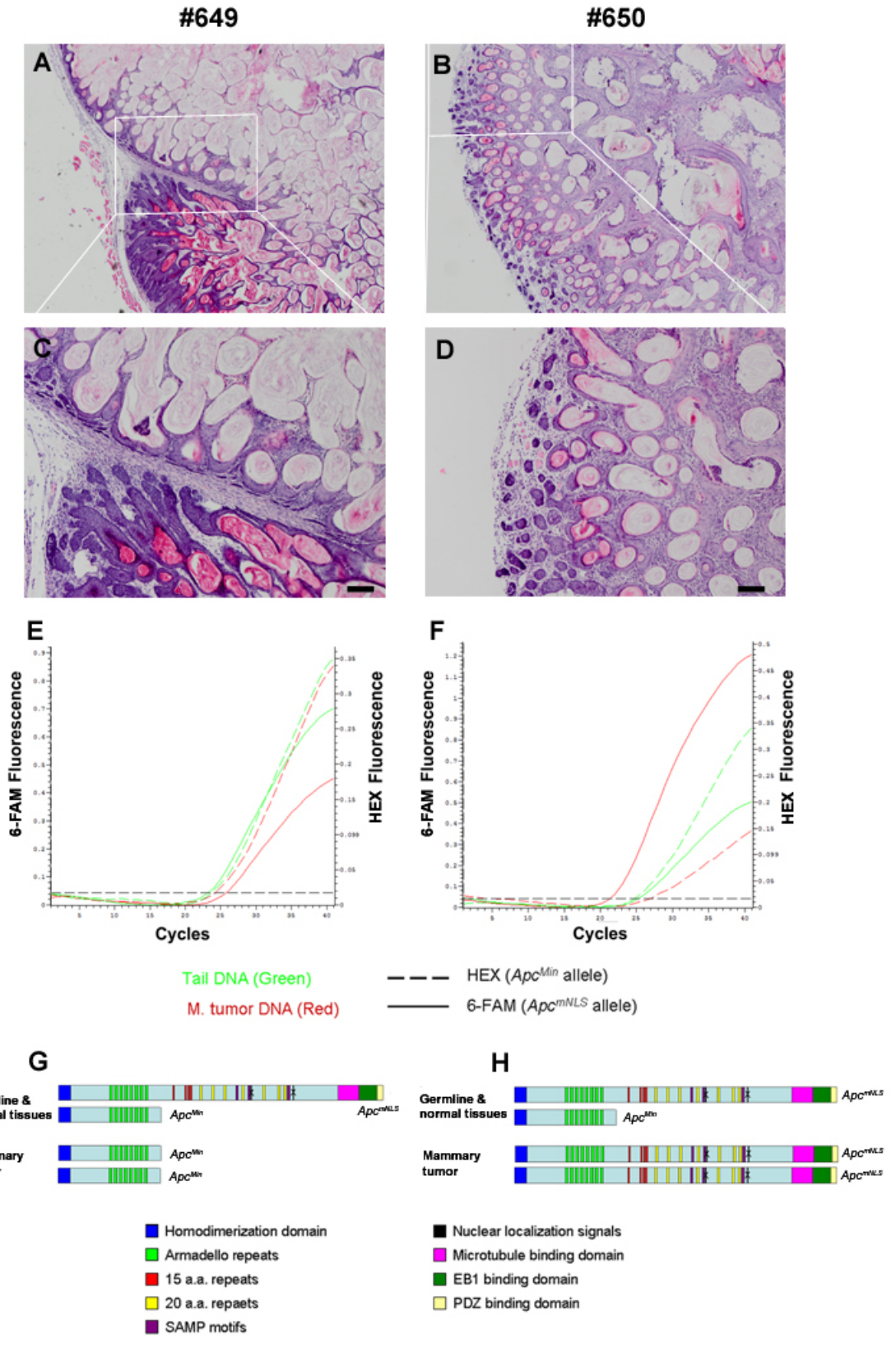
#649
#650
A
B
C
D
E
F
6-FAM Fluorescence
HEX Fluorescence
Cycles
Tail DNA (Green)
M. tumor DNA (Red)
HEX (Apc^Min allele)
6-FAM (Apc^mNLS allele)
G
H
Germline & normal tissues
Mammary tumor
Apc^Min
Apc^mNLS
Homodimerization domain
Armadello repeats
15 a.a. repeats
20 a.a. repeats
SAMP motifs
Nuclear localization signals
Microtubule binding domain
EB1 binding domain
PDZ binding domain

Figure 5. 3: Pathological examination and LOH in mammary tumors from Apc$^{mNLS/Min}$. Hematoxylin and eosin staining of mammary tumors from mouse #649 (A& C) and mouse #650 (B& D). The tumors are squamous cell carcinoma with adenoacanthoma. Note the accumulated keratin in the tumor. Scale bar = 100 μm. E& F show the qPCR results quantifying the *Apc*^{mNLS} (solid lines) and *Apc*^{Min} (dashed lines) alleles from genomic DNA extracted from mammary tumor from mouse #649 (E) and mouse #650 (F) (red) and the tail of the same mouse (green). There is a decrease in the abundance of the *Apc*^{mNLS} allele compared to the *Apc*^{Min} allele in the mammary tumor from mouse # 649 and a decrease in the abundance of the *Apc*^{Min} allele compared to the *Apc*^{mNLS} allele in the mammary tumor from mouse # 650. (G& H) diagrams show the Apc protein structure predicted in normal tissue and in mammary tumors from mouse #649 and # 650 respectively.

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Chapter 6

Characterization and screen for polymorphic loci in the promoter of the *Pla2ga2/Modifier of Min-1* (*Mom-1*) gene in three mouse colonies

Abstract

In this chapter, we show results that define a polymorphic locus in the promoter region of a genetic modifier of Min (*Pla2g2a, Mom-1*) in long-lived mice in our colony. We also show the design of a simple and reliable PCR-based method to screen mouse colonies for this polymorphic *Mom-1* allele. Furthermore, we show that the presence of the polymorphic allele is associated with prolonged longevity in $Apc^{1322T/+}$ mice and with differential expression of the *Pla2g2a* gene.

Introduction

The number of intestinal tumors that develop in different Apc mouse models varies greatly from one laboratory to another. For example, in a C57Bl/6J background, the number of reported polyps in a $Apc^{Min/+}$ mouse, the most characterized Apc mouse model, ranges from 20 to >100 [1]. This inter-laboratory variation represents differences in environment, including diet, sensitivity of polyp detection, as well as genetic variations (see chapter 2 and [2]). In different mouse strains, the number of polyps varies greatly for the same *Apc* mutation. This variation allowed detection of modifier mutations that affect intestinal phenotypes [1]. Identification of modifiers in model organisms allows delineation of the different pathways that contribute to development of a particular phenotype [3]. Several loci have been identified that can alter intestinal polyposis in $Apc^{Min/+}$ mice. These loci were named *modifiers of min* (Mom) [3]. Specific mutations in specific genes have been defined for some, but not all, of these loci [3]. The molecular

mechanisms by which some of these modifiers alter intestinal phenotypes have also been elucidated (see [2] and chapter 2).

The first and most characterized modifier of Min is *Mom-1*, which was discovered in the early 1990s [4]. Mom-1 was linked to a 4 cM region, containing the *Pla2g2a* gene (encoding secreted phospholipase A2 enzyme) on mouse chromosome 4 [5, 6]. MacPhee *et al*. found that mouse strains such as AKR that are resistant to intestinal polyposis driven by Apc^{Min} showed expression of the *Pla2g2a* gene in the intestine, while sensitive mice showed virtually no *Pla2g2a* expression [5]. Cormier *et al*. found that overexpression of *Pla2g2a* gene in a transgenic mouse they made rescues Mom-1 phenotype in $Apc^{Min/+}$ mice and reduces the number of polyps in B6 sensitive strain, confirming that *Pla2g2a* is the candidate gene for Mom-1 phenotype [6]. Although there is no evidence for a role for human *PLA2G2A* in colon cancer [7], it has been shown to play a role in human gastric carcinoma [8]. Apart from an intronic mutation, predicted to result in alternative splicing, no other mutations were reported in the *Pla2g2a* gene [5].

After observing that some $Apc^{Min/+}$ and $Apc^{1322T/+}$ mice in our colonies showed extended life span and reduced intestinal polyp burden, we screened these mice for mutations in known modifiers. We found an allele with polymorphic loci in the promoter of the *Pla2g2a* gene. The presence of this polymorphic allele was associated with increased lifespan in these mice. We also found that this polymorphic allele is associated with a higher amount of the *Pla2g2a* mRNA in the intestinal epithelial cells from C57Bl/6J mice. We developed an easy reliable method to screen our colonies through use of PCR-ARMS primers (amplification refractory mutation system)

Material and methods

Sequencing the *Pla2g2a* gene and other known mutations in *Mom-2*, *Mom-5*, & *Mom*-7mutants

Genomic DNA extracted from mice tails was amplified with primers covering the entire *Pla2g2a* gene, including exons, exon-intron junctions, and ~ 1 kbp of 5' UTR and 3'UTR (table 6. 1). Gel-purified PCR products were sent for sequencing (ACGT Inc.).

Screening for *Pla2g2a* polymorphism

PCR was performed in a 25 µl volume reaction containing 100- 250 ng genomic DNA, 0.4 mM dNTPs (NEB), 15 picomoles of common, wild-type, and *Mom-1* primers (table 6. 1 shows the sequences of these primers) and 0.5 units of *Taq* DNA polymerase. The reaction conditions were 94 ^{o} C for 5 minutes, then 35 cycles of denaturation at 94 ^{o} C for 30 seconds, annealing at 52 ^{o} C for 30 seconds and extension at 68 ^{o} C for 30 seconds followed by a final extension at 68 ^{o} C for 3 minutes.

Measurement of *Pla2g2a* mRNA levels in intestinal epithelial cells

Intestinal epithelial cells from jejunum, ileum, and colon were extracted and used for qRT-PCR with *Pla2g2a*- (table 6. 1) and control *HGPRT*-specific primers, as described in chapter 3.

Table 6. 1 primers used for screening the modifiers and for quantification of *Pla2g2a* mRNA

Primer name	Forward primer	Backward primer	comment
Mom1-1	5'-gtaaggtggctccgtggtaa-3'	5'-catacccaatgccctttttg-3'	Promoter region
Mom1-2	5'-ggcagttggaattcaggaaa-3'	5'-ttgagcctgaaaggaaatgg-3'	
Mom1-3	5'-tcaccacccttttaccaggtc-3'	5'-ctggaaaccactgggacact-3'	
Mom1-4	5'-gtaaggccaccccagttctc-3'	5'-atcttttggccacactctgc-3'	
Mom1-5	5'-cctaaaacagggcacacaca-3'	5'-agtggctgaggatgaccttg-3'	
Mom1-6	5'-gccctctgcagtgtatgaaa-3'	5'tccaagttatgagaacacacacg-3'	
Mom1-7	5'-aggccctcacaagtaaagca-3'	5'-cctggtttttgatggctctc-3'	3'UTR region
Mom-2*	5'-accatctctccagcaccaag-3'	5'-ggcaaaatgagacttaaatgctt-3'	Flanks exon 3 of *Atp5a*
Mom-5*	5'-tgttggaacgtgtttttgga-3'	5'-aatcgcatagatacactgtctgag-3'	
Mom-7*	5'-aacccaggactgcttccttt-3'	5'- ttagaaggcaggagcagagg-3'	
Primers for screening for *Pla2g2a* promoter polymorphisms			
Mom1-com	5'-tgattttgaaacctctcctga-3'		Common primer
Mom1-WT	5'-aaacacacgcagagaattcg-3'		Wild-type-specific primer
Mom1-mut	5'-tcgtatccctgaatgtcttca		Polymorphism-specific primer
Primers for QRT-PCR for *Pla2g2a* mRNA			
Pla2g2a	5'-tacaagcgcctggagaaaag-3'	5'-ggccttatcgcactgacaca-3'	
HGPRT	5'- tgctcgagatgtcatgaagg-3'	5'- tatgtcccccgttgactgat-3'	

We screened for previously described mutations [9-11]

Results

Polymorphisms in the promoter of *Pla2g2a* gene in old Apc$^{Min/+}$ and Apc$^{1322T/+}$ mice

The average lifespan of Apc$^{Min/+}$ mice is around 20 weeks [12], while Apc$^{1322T/+}$ mice die at around 16 weeks of age [13]. We observed that some Apc$^{Min/+}$ and Apc$^{1322T/+}$ mice in our colony lived much longer than the average reported lifespan. We suspected a modifier mutation, and decided to screen for known mutations by sequencing the published modifier genes before searching for a new modifier. We did our initial analysis

on one $Apc^{Min/+}$ mouse that lived for 57 weeks and an $Apc^{1322T/+}$ mouse that lived for 37 weeks. In addition, we used an $Apc^{Min/+}$ mouse that lived for 18 weeks and another $Apc^{1322T/+}$ mouse that became sick and was sacrificed at 18 weeks as the controls. We included DNA from two C57Bl/6J mice purchased directly from Jackson laboratory as additional controls. For Mom-2, Mom-5 or Mom-7 mutations, we screened for the reported mutations in these loci and did not find any of these mutations in any mouse that was screened (data not shown). As there were no reported mutations in the *Pla2g2a* gene (Mom-1), we decided to sequence the entire gene including 1-Kb of the 5' and 3' UTR. We found a polymorphic allele in the heterozygous state in the long-lived $Apc^{Min/+}$ and Apc^{13222T} mice but not in the short-lived mice or in the wild-type mice provided directly from the commercial supplier (figure 6. 1 A- B). We compared the polymorphic sequences to the wild-type sequence and found that the polymorphic region extends from nucleotide -407 to -818 upstream of the first coding nucleotide (figure 6. 1 C).

Development of an easy reliable screening test for the Mom-1 promoter polymorphism

We designed PCR-ARMS primers that could specifically differentiate between the wild-type allele and the polymorphic allele in the same reaction. We designed a common primer in the promoter region that is the same in both the wild-type and polymorphic allele. We designed a primer that is complementary to the polymorphic allele that is different from the wild-type allele in its last two 3' nucleotides, and also contained a mismatch mutation at the invariable nucleotide at position -4 from the 3' end. Introducing this mismatch will reduce primer annealing to the wild-type sequence at its 3' region, preventing non-specific amplification without interfering with annealing of the

same primer to the polymorphic allele. We used the same strategy to design a primer specific for the wild-type allele that does not amplify the polymorphic allele. These primers produce two PCR products, 293-bp for the polymorphic allele and over 600-bp for the wild-type allele, allowing easy discrimination via a 1.5% agarose gel for electrophoresis (figure 6. 2). We validated this method using DNA from both long-lived and wild-type mice. In addition, we screened 10 other mice by use of this PCR method and found that three of them carry the polymorphic allele. We sent the DNA from these 10 mice for sequencing and the results precisely matched the results from the PCR-ARMS protocol.

***Pla2g2a* promoter polymorphisms are associated with prolonged survival of $Apc^{1322T/+}$ mice**

As we developed a reliable test for screening for the presence of the *Pla2g2a* polymorphic allele, we screened Apc^{Min}, Apc^{1322T} and Apc^{mNLS} mouse colonies for the presence of the modifiers. We did this screen mainly to exclude data from mice carrying this polymorphic allele from other experiments, but also to investigate the effect of this polymorphic allele on the phenotype in these mice. We found this polymorphic allele in only a few mice in our Apc^{Min} and Apc^{mNLS} mouse colonies, which made statistical analysis unfeasible. On the other hand, we found that over 50% of the tested mice in our Apc^{1322T} colony carried this polymorphic allele. As most of these mice were euthanized at different time points, compiling their data was not informative. However, we found that 5 $Apc^{1322T/+}$ mice with wild-type *Pla2g2a* alleles and 7 $Apc^{1322T/+}$ mice carrying the polymorphic allele were found dead or sacrificed when they were sick, thus providing overall survival information. Comparing the age of these mice at death, we found that

while $Apc^{1322T/+}$ with the wild-type *Pla2g2a* gene lived for 16.4± 1.9 weeks, $Apc^{1322T/+}$ mice carrying the polymorphic allele lived for 26.3± 2.3 weeks (p= 0.00753), see figure 6. 3.

Mice with the polymorphic *Mom-1* promoter have higher levels of *Pla2g2a* mRNA in intestinal epithelial cells

C57Bl/6J mice carry a virtually null *Pla2g2a* allele [5]. We used qRT-PCR with *Pla2g2a*-specific primers to determine if the polymorphic allele that we identified in long-lived Apc-mutant mice has any effect on the expression level of the gene. We measured *Pla2g2a* mRNA levels in epithelial cells extracted from jejunum, ileum, and colons from 3 wild-type C57Bl/6J mice heterozygous for this polymorphic allele, and from three mice that carry only the wild-type *Pla2g2a* allele. Using *HGPRT* as an internal control, we found that mice heterozygous for the *Pla2g2a* promoter polymorphism had 177-, 31- and 77-fold higher *Pla2g2a* mRNA levels in jejunum, ileum, and colon, respectively than did mice with the wild-type *Pla2g2a* promoter. Figure 6. 4 shows RT-PCR amplification products after 30 cycles using *Pla2g2a*- or *HGPRT*-specific primers separated on a 1.5% gel.

Discussion

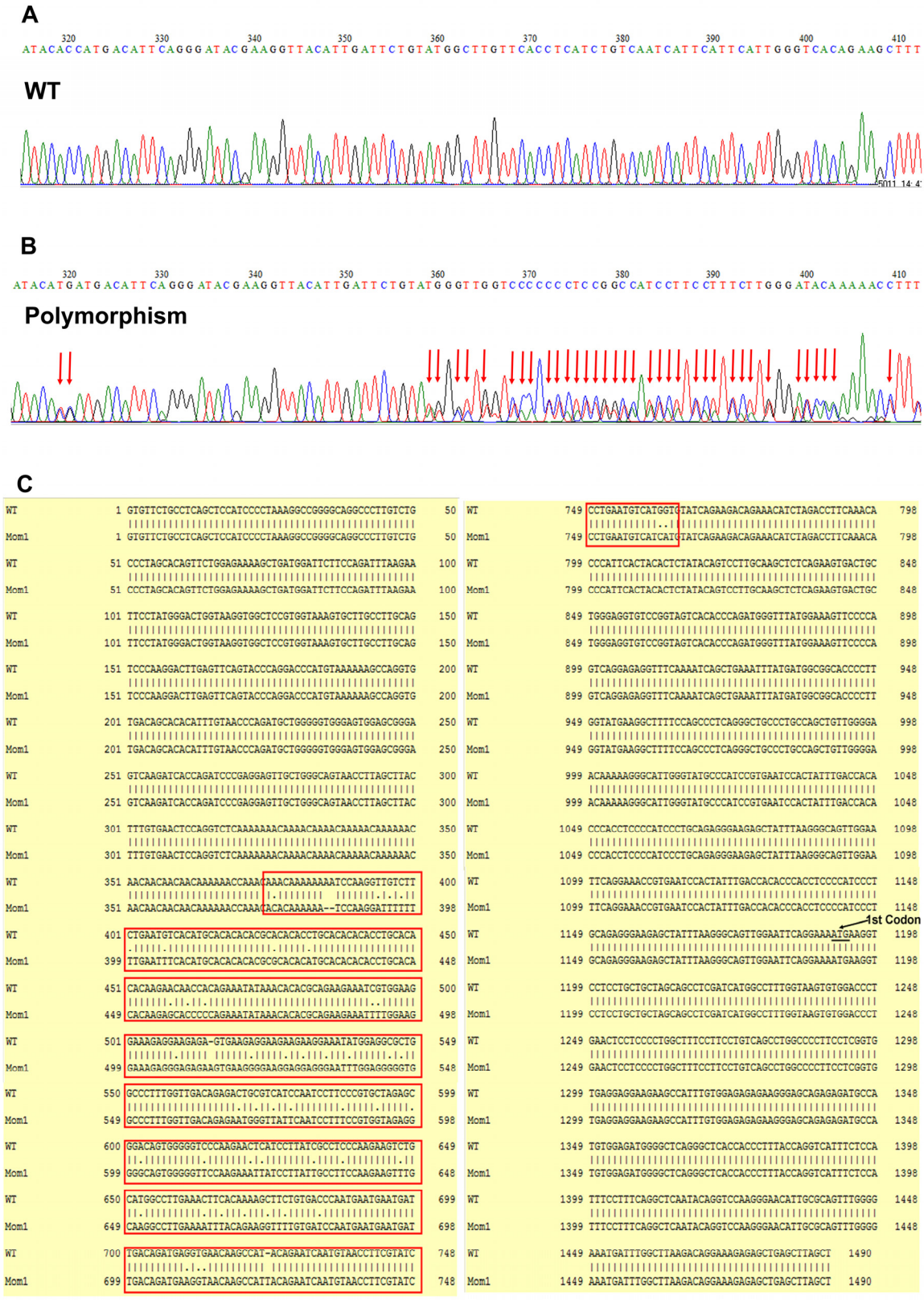
Studying genotypic modifiers allows identification of different pathways contributing to phenotype development [2]. Mom-1 was the first modifier of the intestinal phenotype in $Apc^{Min/+}$ mice to be identified, in 1992 [4]. *Pla2g2a* gene was found to be the candidate gene responsible for Mom-1 phenotype [5, 14]. We found an allele with polymorphic loci in the promoter area of the *Pla2g2a* gene that is associated with prolonged survival in both $Apc^{Min/+}$ and $Apc^{1322T/+}$ mice. To the best of our knowledge,

this is the first report of a specific mutation in this gene that correlates with expression level. Moreover, although Mom1 has been studied in $Apc^{Min/+}$mice, the effect of Mom-1 on the phenotype in $Apc^{1322T/+}$ mice has not been reported. In this study, we showed for the first time that the Mom-1 allele could also prolong the survival of $Apc^{1322T/+}$ mice.

We are not sure if this polymorphic allele emerged as a spontaneous mutation in our colony or from other mouse strains obtained from different vendors. We also do not yet know if the polymorphic allele found in our colony is the same as in different mouse strains with reported resistance to intestinal polyposis. We will continue to screen for this mutation in AKR/J, Ma/MyJ, and CAST/EiJ mouse strains that are known to have the resistant Mom-1 allele [2]. If the *Pla2g2a* allele found in our colony is the same as that present in other mouse strains, it was missed in the previous attempts to sequence this gene [5]. The polymorphic region we found is a relatively long one (~411 bps). It is likely that the primers used in previous attempts to differentially amplify the wild-type and polymorphic alleles hybridized within this polymorphic region and therefore did not amplify this region. In the current study, we used primers that flank a relatively large portion of the promoter (~900 bps) and were fortunate that these polymorphic loci were included in the PCR product.

It has been shown that mice resistant to intestinal polyposis show higher expression of the *Pla2g2a* gene [5]. We found that the polymorphism in the *Pla2g2a* promoter is associated with increased *Pla2g2a* mRNA levels.This correlation, however, needs to be expanded upon experimentally. In the future, we will clone both the wild-type and the polymorphic promoter regions upstream of a reporter construct (e.g. luciferase reporter) and test the effect of these polymorphisms on the reporter expression.

In conclusion, we found a polymorphic locus between -407 to -818 bp upstream of the first exon of the *Pla2g2a* gene. This polymorphic promoter area is correlated with prolonged survival in our mouse colonies. We also found increased expression of the *Pla2g2a* gene in mice harboring this polymorphic promoter region.


A
320 330 340 350 360 370 380 390 400 410
ATACACCATGACATTCAGGGATACGAAGGTTACATTGATTCTGTATGGCTTGTTCACCTCATCTGTCAATCATTCATTCATTGGGTCACAGAAGCTTT
WT
B
320 330 340 350 360 370 380 390 400 410
ATACATGATGACATTCAGGGATACGAAGGTTACATTGATTCTGTATGGGTTGGTCCCCCCCCTCCGGCCATCCTTCCTTTCTTGGGGATACAAAAACCTTT
Polymorphism
C
WT 1 GTGTTCTGCCTCAGCTCCATCCCCTAAAGGCCGGGGCAGGCCCTTGTCTG 50
Mom1 1 GTGTTCTGCCTCAGCTCCATCCCCTAAAGGCCGGGGCAGGCCCTTGTCTG 50
WT 51 CCCTAGCACAGTTCTGGAGAAAAGCTGATGGATTCTTCCAGATTTAAGAA 100
Mom1 51 CCCTAGCACAGTTCTGGAGAAAAGCTGATGGATTCTTCCAGATTTAAGAA 100
WT 101 TTCCTATGGGACTGGTAAGGTGGCTCCGTGGTAAAGTGCTTGCCTTGCAG 150
Mom1 101 TTCCTATGGGACTGGTAAGGTGGCTCCGTGGTAAAGTGCTTGCCTTGCAG 150
WT 151 TCCCAAGGACTTGAGTTCAGTACCCAGGACCCATGTAAAAAAGCCAGGTG 200
Mom1 151 TCCCAAGGACTTGAGTTCAGTACCCAGGACCCATGTAAAAAAGCCAGGTG 200
WT 201 TGACAGCACACATTTGTAACCCAGATGCTGGGGGTGGGAGTGGAGCGGGA 250
Mom1 201 TGACAGCACACATTTGTAACCCAGATGCTGGGGGTGGGAGTGGAGCGGGA 250
WT 251 GTCAAGATCACCAGATCCCGAGGAGTTGCTGGGCAGTAACCTTAGCTTAC 300
Mom1 251 GTCAAGATCACCAGATCCCGAGGAGTTGCTGGGCAGTAACCTTAGCTTAC 300
WT 301 TTTGTGAACTCCAGGTCTCAAAAAAACAAAACAAAACAAAAACAAAAAAC 350
Mom1 301 TTTGTGAACTCCAGGTCTCAAAAAAACAAAACAAAACAAAAACAAAAAAC 350
WT 351 AACAACAACAACAAAAAACCAAACAAACAAAAAAAATCCAAGGTTGTCTT 400
Mom1 351 AACAACAACAACAAAAAACCAAACACACAAAAAA--TCCAAGGATTTTTT 398
WT 401 CTGAATGTCACATGCACACACACGCACACACCTGCACACACACCTGCACA 450
Mom1 399 TTGAATTTCACATGCACACACACGCGCACACATGCACACACACCTGCACA 448
WT 451 CACAAGAACAACCACAGAAATATAAACACACGCAGAAGAAATCGTGGAAG 500
Mom1 449 CACAAGAGCACCCCCAGAAATATAAACACACGCAGAAGAAATTTTGGAAG 498
WT 501 GAAAGAGGAAGAGA-GTGAAGAGGAAGAAGAAGGAAATATGGAGGCGCTG 549
Mom1 499 GAAAGAGGGAGAGAAGTGAAGGGGAAGGAGGAGGAAGGAATTTGGAGGGGGTG 548
WT 550 GCCCTTTGGTTGACAGAGACTGCGTCATCCAATCCTTCCCGTGCTAGAGC 599
Mom1 549 GCCCTTTGGTTGACAGAGAATGGGTTATTCAATCCTTTCCGTGGTAGAGG 598
WT 600 GGACAGTGGGGGTCCCAAGAACTCATCCTTATCGCCTCCCAAGAAGTCTG 649
Mom1 599 GGGCAGTGGGGGTTCCAAGAAATTATCCTTATTGCCTTCCAAGAAGTTTG 648
WT 650 CATGGCCTTGAAACTTCACAAAAGCTTCTGTGACCCAATGAATGAATGAT 699
Mom1 649 CAAGGCCTTGAAAATTTACAGAAGGTTTTGTGATCCAATGAATGAATGAT 698
WT 700 TGACAGATGAGGTGAACAAGCCAT-ACAGAATCAATGTAACCTTCGTATC 748
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WT 749 CCTGAATGTCATGGTGTATCAGAAGACAGAAACATCTAGACCTTCAAACA 798
Mom1 749 CCTGAATGTCATCATGTATCAGAAGACAGAAACATCTAGACCTTCAAACA 798
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Mom1 799 CCCATTCACTACACTCTATACAGTCCTTGCAAGCTCTCAGAAGTGACTGC 848
WT 849 TGGGAGGTGTCCGGTAGTCACACCCAGATGGGTTTATGGAAAGTTCCCCA 898
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WT 899 GTCAGGAGAGGTTTCAAAATCAGCTGAAATTTATGATGGCGGCACCCCTT 948
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WT 949 GGTATGAAGGCTTTTCCAGCCCTCAGGGCTGCCCTGCCAGCTGTTGGGGA 998
Mom1 949 GGTATGAAGGCTTTTCCAGCCCTCAGGGCTGCCCTGCCAGCTGTTGGGGA 998
WT 999 ACAAAAAGGGCATTGGGTATGCCCATCCGTGAATCCACTATTTGACCACA 1048
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WT 1099 TTCAGGAAACCGTGAATCCACTATTTGACCACACCCACCTCCCCATCCCT 1148
Mom1 1099 TTCAGGAAACCGTGAATCCACTATTTGACCACACCCACCTCCCCATCCCT 1148
1st Codon
WT 1149 GCAGAGGGAAGAGCTATTTAAGGGCAGTTGGAATTCAGGAAAATGAAGGT 1198
Mom1 1149 GCAGAGGGAAGAGCTATTTAAGGGCAGTTGGAATTCAGGAAAATGAAGGT 1198
WT 1199 CCTCCTGCTGCTAGCAGCCTCGATCATGGCCTTTGGTAAGTGTGGACCCT 1248
Mom1 1199 CCTCCTGCTGCTAGCAGCCTCGATCATGGCCTTTGGTAAGTGTGGACCCT 1248
WT 1249 GAACTCCTCCCCTGGCTTTCCTTCCTGTCAGCCTGGCCCCTTCCTCGGTG 1298
Mom1 1249 GAACTCCTCCCCTGGCTTTCCTTCCTGTCAGCCTGGCCCCTTCCTCGGTG 1298
WT 1299 TGAGGAGGAAGAAGCCATTTGTGGAGAGAGAAGGGAGCAGAGAGATGCCA 1348
Mom1 1299 TGAGGAGGAAGAAGCCATTTGTGGAGAGAGAAGGGAGCAGAGAGATGCCA 1348
WT 1349 TGTGGAGATGGGGCTCAGGGCTCACCACCCTTTACCAGGTCATTTCTCCA 1398
Mom1 1349 TGTGGAGATGGGGCTCAGGGCTCACCACCCTTTACCAGGTCATTTCTCCA 1398
WT 1399 TTTCCTTTCAGGCTCAATACAGGTCCAAGGGAACATTGCGCAGTTTGGGG 1448
Mom1 1399 TTTCCTTTCAGGCTCAATACAGGTCCAAGGGAACATTGCGCAGTTTGGGG 1448
WT 1449 AAATGATTTGGCTTAAGACAGGAAAGAGAGCTGAGCTTAGCT 1490
Mom1 1449 AAATGATTTGGCTTAAGACAGGAAAGAGAGCTGAGCTTAGCT 1490

Figure 6. 1: Polymorphic region in the *Pla2g2a* gene promoter in long-lived Apc$^{Min/+}$ and Apc$^{1322T/+}$ mice. A representative chromatograph showing the sequence in the promoter area of the *Pla2g2a* gene in wild-type mice (**A**) and in a long-lived Apc$^{Min/+}$ mouse (**B**). The polymorphic sequences result in double peaks and are marked by red arrows. (**C**) Alignment of the polymorphic (Mom1) and the wild-type (WT) sequences. The polymorphic area is marked by the red rectangles while the first codon is marked by an arrow.

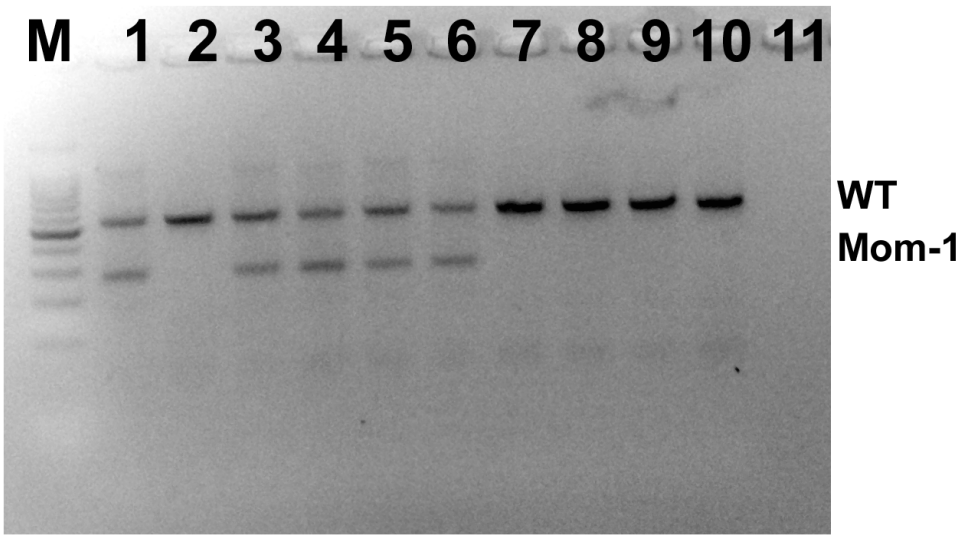

Figure 6. 2: Development of an easy and reliable screen for *Pla2g2a* gene promoter polymorphisms. An example of PCR products separated on a 1.5% agarose gel and stained with ethidium bromide for mice heterozygous for? the polymorphic *Pla2g2a* promoter allele (samples 1, 3, 4, 5 & 6) and mice homozygous for the C57Bl/6J wild-type allele (samples 2, 7, 8, 9 & 10). First lane (M) is a 100-bp ladder while the last lane is a negative control with no template.

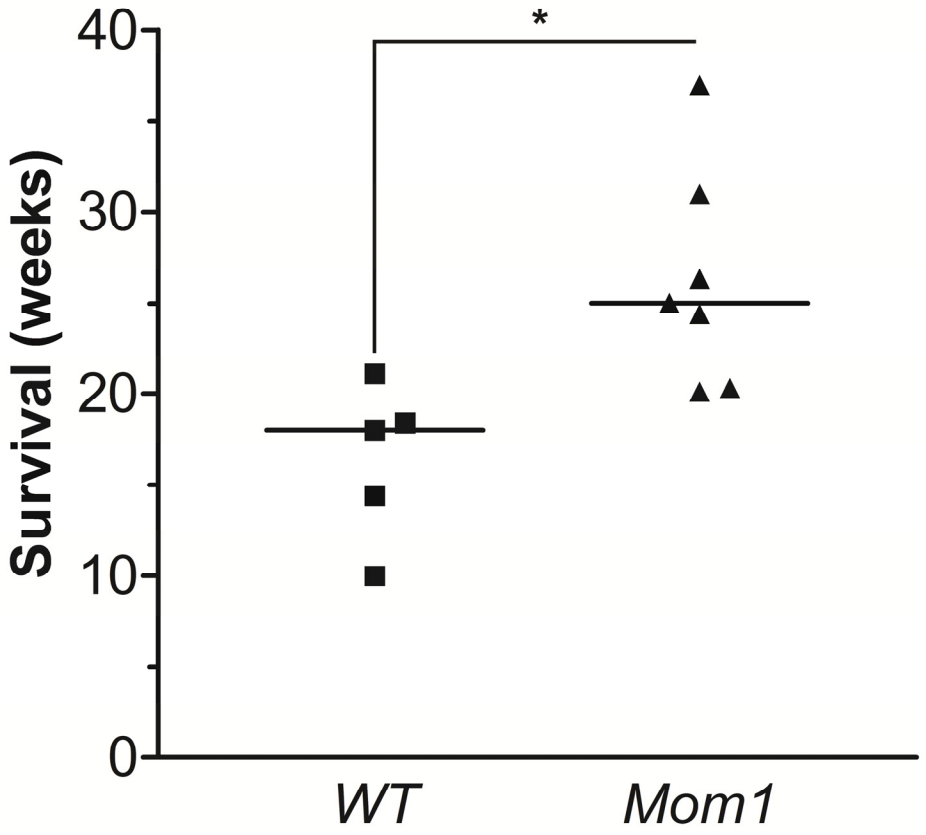

Figure 6. 3: *Pla2g2a* gene with polymorphic allele is associated with prolonged survival of Apc$^{1322T/+}$ mice. Scatter plot for the recorded survival of Apc$^{1322T/+}$ mice with wild-type (WT) and with polymorphic *Pla2g2a* promoter (*Mom1*). The lines represent the median survivals of both groups while (*) indicates $p< 0.05$.

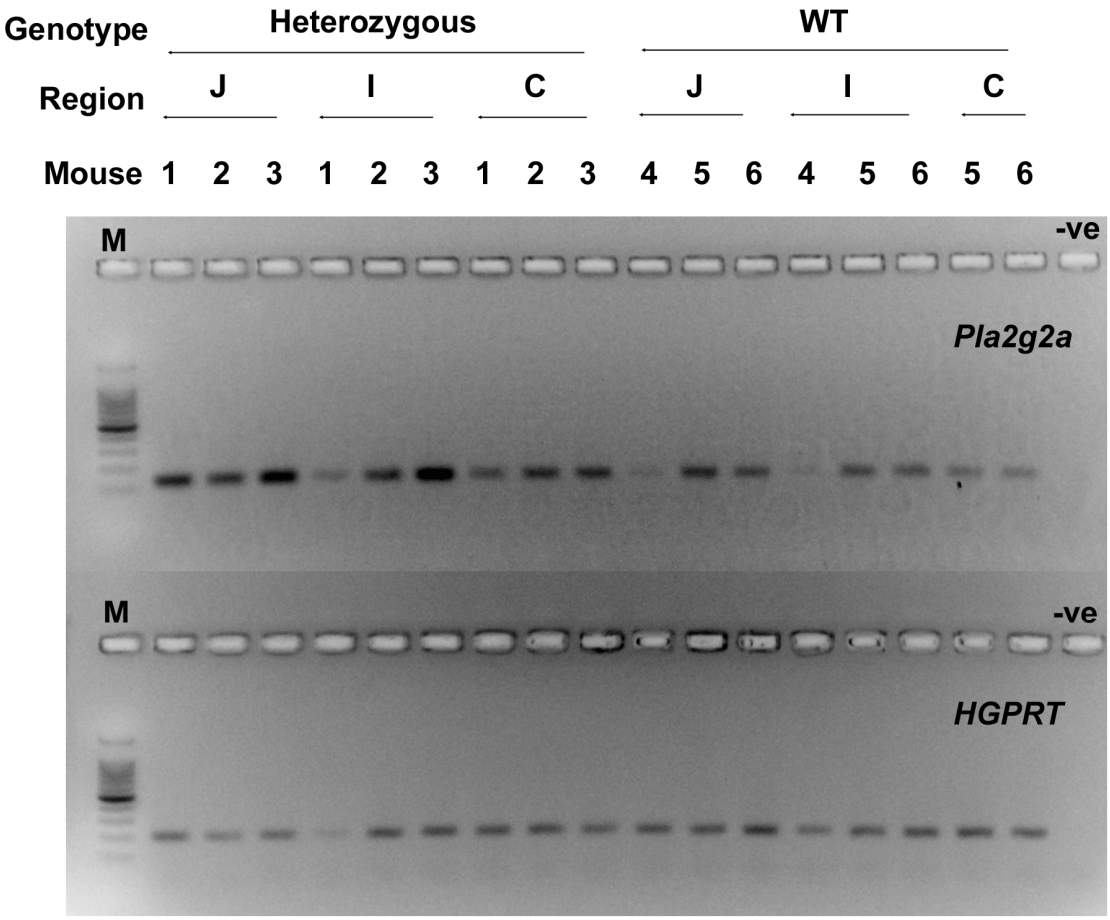

Figure 6. 4: Mice heterozygous for the *Pla2g2a* polymorphic promoter allele have higher levels of mRNA in their small and large intestinal epithelial cells. RT-PCR products using *Pla2g2a*- (upper) and *HGPRT*-specific (lower) primers and mRNA isolated from jejunum (J), ileum (I), and colon epithelial cells from mice heterozygous to the polymorphic promoter allele and from wild-type mice. The first lane is a 100-bp ladder (M) while the last lane is a negative control sample without template (-ve).

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Chapter 7

Induction of the heat-shock response upregulates the tumor suppressor APC and alters intestinal tumorigenesis in mice

Abstract

Mutation of the tumor suppressor gene Adenomatous Polyposis Coli (*APC*) is considered an initiating event in the development of most intestinal tumors. Although much effort has been spent determining functions of the APC protein, to date little is known about the mechanisms that regulate cellular APC levels. Here we report that in cultured cells, induction of a heat-shock response, via heat or compounds such as the HSP90 inhibitor 17-AAG, resulted in increased levels of APC. A novel non-toxic small molecule, KU-32, also induced a heat-shock response and led to increased APC levels in both cultured cells and mice. To investigate the effect of induction of the heat-shock response and elevation of Apc level in intestinal tumorigenesis, we performed a series experiments in mice with KU-32 and 17-AAG. We tested these compounds on two mouse models with mutations in Apc, the $Apc^{Min/+}$ and the $Apc^{1322T/+}$ mice. In both cases, a moderate dose of either drug did not change tumor burden, but surprisingly altered the location of intestinal polyps. A higher dose of KU-32 actually increased tumor size and number in $Apc^{Min/+}$ mice. In a third mouse model where colonic tumors are induced via administration of the mutagen azoxymethane and dextran sodium sulfate (AOM-DSS model) rather than through germline *Apc* mutation, KU-32 reduced both tumor incidence and multiplicity. We conclude that induction of the heat-shock response affects intestinal tumorigenesis in germline *Apc*-mutant and colitis-induced tumor models.

Introduction

Colorectal cancer is one of the leading causes of cancer-related death in the United States [1]. Loss of the tumor suppressor functions of Adenomatous Polyposis Coli (APC) is the initiating step in most cases of colorectal cancer [2]. APC is a large multidomain protein (2843 amino acids) that has been implicated in many cellular functions in the cells, including proliferation, migration, cytoskeletal regulation, apoptosis, and chromosomal segregation [3]. APC is best known for its role in opposing Wnt signaling-induced cellular proliferation by forming a complex that phosphorylates the oncoprotein β-catenin and targets it for proteasomal degradation. APC mutation in colorectal cancers results in accumulation and nuclear translocation of β-catenin, to induce expression of Wnt target genes that drive cellular proliferation [3]. Control of cellular APC level is not completely understood, but seems to occur at the transcriptional, translational, and posttranslational levels (see chapter 1).

To facilitate studying Apc, several mouse models have been made [4]. The first and most characterized model is the $Apc^{Min/+}$ mouse. $Apc^{Min/+}$ mice have a heterozygous nonsense mutation resulting in a truncated 850-amino acid Apc protein. $Apc^{Min/+}$ mice develop between 20 and 100 intestinal polyps predominantly in the small intestine [5]. It has been shown that loss of the wild-type allele (LOH) is required for polyp formation [6]. Another mouse model that was recently generated is the $Apc^{1322T/+}$ mouse. In $Apc^{1322T/+}$ mice, a truncation mutation results in a longer Apc protein (1322 amino acids) than in $Apc^{Min/+}$ mice. The mutation in $Apc^{1322T/+}$ mice is more close to mutations detected in human colorectal cancer. $Apc^{1322T/+}$ mice develop intestinal tumors, though with a different distribution. Most polyps in $Apc^{1322T/+}$ mice are in the proximal part of the small

intestine, while in $Apc^{Min/+}$ mice, tumors tend to be more distal, in the ileal region [7]. As in $Apc^{Min/+}$ mice, intestinal polyps in $Apc^{1322T/+}$ mice show LOH. Since Apc^{1322T} protein retains the first 20-amino acid repeat domain that can bind to β-catenin while this domain is lost in $Apc^{Min/+}$ mice, it is not surprising that Wnt signaling activity is not as strongly upregulated in polyps from $Apc^{1322T/+}$ mice relative to those from $Apc^{Min/+}$ mice [8].

Chronic inflammation in the colon (chronic colitis) is a major risk factor for the development of colorectal cancer. Patients with inflammatory bowel disease (Crohn's disease and ulcerative colitis) have a significant increased risk of developing an aggressive form of colorectal cancer (colitis-associated colorectal cancer) [9]. Treatment of mice with Azoxymethane- Dextran Sodium Sulfate (AOM-DSS) has been described as a model of colitis-associated colorectal cancer [10]. In this model, colon cancer is initiated in mice by injecting mice with the mutagen AOM followed by induction of chronic colitis through cycles of DSS in the drinking water [10]. Unlike mouse models with *Apc*-germline mutations, which develop tumors mainly in the small intestine, AOM-DSS-treated mice develop colonic tumors [10]. In the AOM-DSS mouse model, *Apc* is usually not mutated, but instead, mutations occur in β-catenin that prevent its phosphorylation and proteasomal degradation (stabilizing mutations). Wnt signaling is thereby activated to initiate colonic tumorigenesis [11].

The heat-shock response describes induction of specific cellular proteins (heat-shock proteins/ stress proteins) to deal with sudden changes in the cellular environment. Heat-shock proteins (molecular chaperones) bind to damaged proteins to assist in refolding and preventing toxic aggregates [12]. Even in the absence of stress, heat-shock proteins help fold many proteins and translocate some proteins to different organelles,

including the mitochondria [12]. The most abundant heat-shock protein in the cell is HSP90. HSP90 is a target of several anticancer drugs that are currently in clinical trials [13]. As HSP90 is critical for the proper folding and function of many proteins involved in cellular transformation, HSP90 serves as a potential target to decrease survival of cancer cells. HSP90 inhibitors interfere with proper folding of HSP90 client proteins, resulting in their ubiquitination and degradation [14].

Here we show that induction of the heat-shock response either through application of the commercially-available HSP90 inhibitor 17-AAG, or by heating the cells, increases the cellular level of APC in colon cancer cell lines. We also show that induction of the heat-shock response by means of the novel non-toxic drug KU-32 upregulates the level of APC both *ex vivo* in cultured colon cancer cells and *in vivo* in mouse intestinal epithelial cells. The increase in APC level in cultured cells is associated with reduced β-catenin transcriptional activity. To test the effect of induction of the heat-shock response and upregulation of Apc on intestinal tumorigenesis, we treated $Apc^{Min/+}$ mice with 2 different doses of KU-32 and monitored the effect of the compound on intestinal polypogenesis. We also treated $Apc^{Min/+}$ mice with 17-AAG to induce the heat-shock response with a known HSP90 inhibitor, and examined changes in intestinal tumorigenesis. Moreover, we tested KU-32 on two additional mouse models: $Apc^{1322T/+}$ and AOM-DSS mice. We found that, the higher but not the lower dose of KU-32 increases the polyp burden in $Apc^{Min/+}$ mice. The lower dose of KU-32 does not affect polyp burden in $Apc^{1322T/+}$ mice. 17-AAG does not affect polyp number in all intestinal regions but decreases polyp size in the upper (stomach and duodenum) and lower (colon) gastrointestinal tract of $Apc^{Min/+}$ mice. However, KU-32 and 17-AAG changes the

distribution of intestinal polyps in $Apc^{Min/+}$ and $Apc^{1322T/+}$ mice. Finally, we found that KU-32 reduces colonic tumor incidence and multiplicity in AOM-DSS treated mice. We concluded that induction of the heat-shock response increases APC level and impacts intestinal tumorigenesis.

Materials and methods

Mouse Husbandry

Mice were maintained in the Animal Care Unit at the University of Kansas according to animal use statement number 137-01. The research complied with all relevant federal guidelines and institutional policies. Mice were fed *ad libitum* with Purina Lab Diet 5001. $Apc^{Min/+}$ mice were purchased from The Jackson Labs and were maintained by breeding males with C57BL/6J females. $Apc^{1322T/+}$ mice were provided as a generous gift from Ian Tomlinson's lab, University of Oxford, and were maintained by breeding $Apc^{1322T/+}$ males with C57Bl/6J females.

Mouse genotyping

After weaning, mouse pups were tagged with a metal ear tag. Each mouse pup was genotyped for Apc^{Min} or Apc^{1322T} alleles using isolated tail DNA and PCR according to published protocols [7; 15].

Counting the polyps and measuring polyp diameters

For each mouse, after euthanization, the GI tract from the stomach to the anal canal was dissected, opened longitudinally, and fixed in 10% buffered formalin. Using a dissecting microscope, an investigator blind to the treatment protocol examined the intestinal luminal surface for polyps. Polyp diameter was measured with the aid of a

dissection microscope (Leica, MZ8) equipped with an eyepiece graticule and calibrated to a 50-mm scale stage micrometer with 0.1 and 0.01-mm gradation [16].

Treatment of cultured cells

HCT116βW and HT29 colon cancer cell lines were maintained on McCoy's 5A medium with 10% fetal bovine serum. When the cells were 60-80% confluent, media was removed, and fresh media containing either the drug or the drug vehicle (DMSO for 17-AAG and MG132, and Captisol for KU-32) was added. For thermal treatment, fresh media was added to cells when they were 60-80% confluent. The cells were transferred to a preheated incubator with 5% CO_2 at 42°C for different times.

Luciferase reporter assay for β-catenin transcriptional activity

β-catenin transcriptional activity was measured using the standard Super TOPFlash luciferase reporter system, in which the firefly *luciferase* gene is transcribed under the control of a minimal promoter and 16 TCF/LEF1 binding sites [17]. A DNA construct with mutations in the TCF/LEF1-binding sites (FOPFlash) was used as a control. We transfected HCT116 cells with 1.8 μg of either the TOPFlash or FOPFlash DNA construct and 0.2 μg Renilla *luciferase* as a transfection control. After 24 hours, we treated the cells with either 1.5 μM KU-32 or the drug vehicle (captisol) for 24 hours. The cells were harvested in 1X reporter buffer, and firefly and Renilla luciferase activities were measured by use of the 'Dual Luciferase Reporter Assay System" (Promega) according to the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase acitivty. The normalized firefly TOPFlash luciferase activity was then normalized to normalized firefly FOPFlash luciferase in KU-32-treated

cells and was compared to the normalized firefly TOPFlash activity in captisol-treated cells.

Western blot of total cell lysates from cultured and mouse intestinal epithelial cells

After washing twice in cold PBS, cultured cells were harvested in 1X reporter lysis buffer (Promega) with protease inhibitors. Intestinal epithelial cells were isolated as described in Chapter 3 [16], and cells were lysed in 1X reporter buffer (Promega) with protease inhibitors. The cultured and intestinal epithelial cells were heated to 95°C for 3 minutes in 1X loading buffer (15% glycerol, 1% SDS and 3% 2-mercaptoethanol, pH 6.8) prior to protein separation via SDS-PAGE on a 7% polyacrylamide gel, and transferred to nitrocellulose. We used rabbit anti-APC M2 (1:3000) [18], mouse monoclonal anti-β-actin (1:5000, Sigma), mouse monoclonal anti-GAPDH (1:1000, Sigma) and mouse monoclonal anti-HSP70 (1:1000, BD transduction laboratories). The following secondary antibodies were diluted as indicated: HRP goat anti-mouse (1:10-25,000, Zymed) and HRP goat anti-rabbit (1:10-25,000, Bio-Rad). Band analysis was conducted with Kodak ID Image Analysis Software, with protein levels normalized to β-actin or GAPDH.

Dissection and weighing of liver and spleens

After being euthanized by means of CO_2, $Apc^{mNLS/Min}$, $Apc^{Min/+}$, and $Apc^{+/+}$ mice were weighed. The livers and spleens were removed, washed in PBS, weighed, and fixed in 10% saline-buffered formalin overnight, then in 70% ethanol.

Complete blood count analysis

Immediately after euthanasia, blood (0.5 - 1 ml) was drawn from the mouse via intra-cardiac puncture using a 20 G heparinized needle. The blood was sent to “Physician

Reference Laboratory" (Overland Park, KS) for complete and differential blood count (CBC) analysis.

Results

Induction of a heat-shock response increases APC level in colon cancer cell lines

To test the requirement of HSP90 in stabilization of APC, we treated two colon cancer cell lines (HCT116 βW with wild-type full-length APC and HT29 with truncated APC) with HSP90 inhibitor 17-AAG. We reasoned that if APC is an HSP90-client protein, inhibiting this molecular chaperone using 17-AAG would result in reduced cellular APC levels. However, we found that the levels of both full-length APC in HCT116 βW and truncated APC in HT29 cells were increased (figure 7.1). These results argue against a role of HSP90 in stabilizing APC. It has been suggested that APC is degraded by the ubiquitin-proteasome pathway [19]. To test if the observed increase in APC level in 17-AAG-treated cells involves the proteasome pathway, we treated the same cell lines with 17-AAG plus the proteasome inhibitor MG132. As expected, we found that MG132 increases the cellular level of APC. However, simultaneous treatment of the cells with both drugs resulted in a greater increase in APC level than with either treatment alone (figure 7.1). We conclude that 17-AAG and MG132 work through two different pathways. One pathway that is induced by 17-AAG is the heat-shock response (see chapter 1, figure 1.4). Inhibition of HSP90 using 17-AAG has been shown to result in destabilizing the complex containing HSP90 and heat-shock factor 1 (HSF1). Free HSF1 goes to the nucleus, forms a homotrimer, and undergoes phosphorylation. Nuclear HSF1 binds to a heat-shock response element (HSE) in the promoters of inducible heat-shock genes, to induce their expression [13]. MG132 also induces the heat-shock

response by inhibiting the proteasome and increasing the level of unfolded proteins. Unfolded proteins bind to HSP90, which increases the level of free HSF1, which in turn induces the heat-shock response [12]. Taken together, we hypothesized that the increase in APC level in 17-AAG-treated cells occurs through induction of a heat-shock response. Supporting this hypothesis, we found that the cellular level of the HSF1-inducible heat-shock protein HSP70 is increased in both 17-AAG- and MG132-treated cells (figure 7.1). In addition, we induced the heat-shock response in HCT116βW and HT29 cells by incubating the cells at 42°C for different durations. We found that APC levels are increased in these heated cells (figure 7.2). We concluded that induction of the heat-shock response increases the cellular APC level in colon cancer cell lines.

A novel small molecule induces a heat-shock response and increases cellular APC level both in cultured cells and in mouse intestinal epithelial cells

Mutations in *APC* initiate colorectal carcinogenesis [2]. We hypothesized that upregulation of APC through induction of the heat-shock response could affect intestinal tumor initiation and/or progression. To test this possibility, we examined a small molecule, KU-32, formulated by Dr. Brian Blagg at the University of Kansas Department of Medicinal Chemistry. KU-32 induces the heat-shock response, but is 10,000 times less toxic than is 17-AAG [20]. In addition, studies in mice have shown that KU-32 has favorable pharmacological properties, such as stability in solution, and nearly complete absorption after an oral dose [20].

First, we tested the effect of KU-32 on APC cellular levels by treating cultured colon cancer cells with 1.5 μM KU-32 for 24 hours. We found that KU-32 induced the

heat-shock response, as shown by upregulation of HSP70 level and increased concentrations of APC (figure 7.3 A-B).

The best-known function of APC is to antagonize the Wnt signaling pathway by targeting β-catenin for proteasome-mediated destruction [21]. If APC induced by KU-32 actively inhibits β-catenin, cells treated with KU-32 should show reduced β-catenin activity. Using standard reporter constructs to estimate β-catenin activity as a transcription co-factor, we determined that cells treated with KU-32 displayed a 25% reduction in β-catenin activity compared to cells treated with vehicle alone (figure 7.3 C). We conclude that KU-32 leads to reduced β-catenin signaling activity, most likely through upregulation of APC.

To test whether KU-32 alters the level of Apc in intestinal epithelial cells of a whole organism, we administered intraperitoneal injections of 30mg/kg KU-32 to C57BL/6 mice. Sibling mice were injected with the drug vehicle only. Four mice were sacrificed at each of two time points post injection. Relative Apc levels were determined in whole-cell lysates of epithelial cells isolated from the small and large intestines of KU-32-treated mice. APC levels were higher in intestinal epithelial cells from mice treated with KU-32 compared to mice treated with vehicle alone for 24 hours (figure 7.3 D-E). By 48 hours post injection, Apc levels remained elevated in the jejunum and to a lesser extent, in the ileum (figure 7.3 F-G). Together, these data provide evidence that KU-32 increases the amount of APC in intestinal epithelial cells grown in culture and in mice. To determine the minimal dose of KU-32 that could increase APC levels in mouse intestinal epithelial cells, mice were injected with doses of KU-32 ranging from 0.3 to 30 mg/kg, and the change of Apc level was assessed 24 hours later.. We found that the

smallest KU-32 dose that could increase Apc level in mouse intestinal epithelial after 24 hours is 7.5 mg/kg (figure 7.3 H). Next, we treated mice with this dose of KU-32 (7.5 mg/kg) for 7, 9, and 14 days in order to determine the duration of Apc increase. We found that Apc levels remained elevated after one week in intestinal epithelial cells. We conclude that 7.5 mg/kg weekly is the smallest dose that maintains an elevated level of Apc in mouse intestinal epithelial cells.

KU-32 alters intestinal tumorigenesis in $Apc^{Min/+}$ mice

KU-32 increases the level of APC and reduces β-catenin transcriptional activity in cultured cells. As KU-32 also upregulates Apc in mouse intestinal epithelial cells, we expected that this novel non-toxic compound could reduce intestinal tumorigenesis in $Apc^{Min/+}$ mice. The $Apc^{Min/+}$ mouse is the best characterized Apc mouse model. These mice have a nonsense mutation in the *Apc* gene that results in a truncated protein at amino acid 850 (see chapter 2) [22]. We used two different KU-32 treatment protocols in $Apc^{Min/+}$ mice. In the first protocol, we gave 9 $Apc^{Min/+}$ mice intraperitoneal injections of 30mg/kg KU-32 every 2 weeks starting at 2 weeks old. We also injected 10 other $Apc^{Min/+}$ mice with the drug vehicle only, and these mice served as a control group. After 7 doses (14 weeks), mice were euthanized and the entire gastrointestinal tract from the stomach to the anus was dissected to determine the number and size of the polyps. In KU-32-treated mice, there was a significant reduction in size of polyps found in the jejunum. However, KU-32-treated mice had more polyps, on average, in all intestinal tissues examined, and polyps of the ileum and colon were significantly larger in the KU-32 treated group (figure 7.4 A-D). In addition to the effect on the intestinal polyp burden, KU-32 also significantly changed the distribution of polyps in $Apc^{Min/+}$ mice. There was a

distal shift in intestinal polyps, increasing the ratio of ileal polyps from 55% in the control group to 71% in KU-32-treated mice (figure 7.4 E).

Second, we treated 10 $Apc^{Min/+}$ mice with intraperitoneal injections of 7.5 mg/ kg KU-32 every week starting at the age of 6 weeks, and ending at 10 weeks. We also treated 9 $Apc^{Min/+}$ mice with the drug vehicle as a control group. We reasoned that this short KU-32 treatment protocol could address some caveats in the previous study. As the mouse intestine is not fully developed by the age of 2 weeks, using KU-32 at this young age may influence the development of the intestine. Therefore, we began KU-32 injections in older mice (6 weeks) and used the smallest dose of KU-32 that could increase Apc level in mouse intestinal epithelial cells (figure 7.3 H-I). The end point was set to 10 weeks, at which time the polyps are easily detectable in the intestinal tract of the mice. We also weighed spleens and livers, and collected blood for complete and differential blood count from the mice in both groups. We found that there was no significant change in either polyp multiplicity or polyp size in $Apc^{Min/+}$ mice treated with the short KU-32 regimen relative to the control group (figure 7.5 A-D). However, we noticed the same trend of altered polyp distribution in different intestinal regions (figure 7.5 E). We did not find significant differences in spleen weight, liver weight, or blood count in KU-32-treated mice relative to the control group (data not shown). We conclude that KU-32 influences intestinal tumorigenesis in $Apc^{Min/+}$ mice.

17-AAG changes intestinal polyp size and distribution in $Apc^{Min/+}$ mice

To investigate whether the effects of KU-32 on $Apc^{Min/+}$ polyps are the result of heat-shock response induced by the compound, we tested the effects of 17-AAG on intestinal tumorigenesis in $Apc^{Min/+}$ mice. 17-AAG is an HSP90 inhibitor that induces the

heat-shock response by releasing HSF1 from the cytoplasmic complex that sequesters it (see chapter 1; figure 1-4) [23]. We gave 9 $Apc^{Min/+}$ mice intraperitoneal injections of 50mg/kg 17-AAG three times a week starting at 6 weeks of age. This dose appears to be well-tolerated in mice [24]. A control group of 10 $Apc^{Min/+}$ mice was included, and received injections of the drug vehicle. After 4 weeks, the mice of both groups were sacrificed, and the intestinal polyps in the 17-AAG-treated and control groups were counted and measured. Liver and spleen weights were recorded, and complete and differential blood counts were measured as described previously. We did not find a significant change in the polyp multiplicity (figure 7.6 A-C), liver weight, spleen weight, or hematological profile in both groups. There was also no significant difference in the diameter of polyps in jejunum or ileum. However, 17-AAG significantly reduced the average sizes of polyps in the proximal (stomach and duodenum) and distal gastrointestinal tract (colon) from treated mice relative to the control group (figure 7.6 D). As observed with KU-32, 17-AAG significantly altered polyp distribution in $Apc^{Min/+}$ mice by increasing the percentage of ileal polyps from 51% in the control group to 64% in the treated group (figure 7.6 E). We conclude that both 17-AAG and KU-32 alter intestinal polyp distribution in $Apc^{Min/+}$ mice.

KU-32 changes polyp distribution in $Apc^{1322T/+}$ mice

APC is a large multi-domain protein and has many cellular roles [21]. The best characterized function of APC is to antagonize Wnt signaling by targeting the oncoprotein β-catenin for proteasomal degradation. Most colon cancer-associated-mutations in *APC* result in truncation of the C-terminal half of the protein, retaining the first 20-amino acid repeat, which has the ability to bind β-catenin [3]. The Apc mutation

in $Apc^{Min./+}$ mice results in a shorter truncated protein (850 amino acids) that is rarely seen in human disease. This first 20-amino acid repeat region is absent in the truncated Apc^{Min} protein [22]. We tested KU-32 in another mouse model, the $Apc^{1322T/+}$ mouse. We reasoned that as the mutation in $APC^{1322T/+}$ mice more closely resembles the mutations in human colorectal cancer [7], testing KU-32 in these mice may provide more relevant information for the human disease. In addition, we have shown that upregulation of APC is associated with decreased β-catenin transcriptional activity in HCT116 βW colon cancer cell line. As $Apc^{1322T/+}$ mice retain the first 20-amino acid repeats, Apc upregulation in these mice could result in reduction of β-catenin transcriptional activity and decrease intestinal tumorigenesis. Comparing the effects of KU-32 on $Apc^{Min/+}$ versus $Apc^{1322T/+}$ mice should help understand the effect of heat-shock response in intestinal tumorigenesis.

We injected 10 $Apc^{1322T/+}$ mice with 7.5 mg/kg KU-32 every week starting at the age of 4.5 weeks. Ten other $Apc^{1322T/+}$ mice were treated with the drug vehicle only, and served as a control group. As described in chapter 6, we found a polymorphic allele in the promoter of *Modifier of Min 1 (Mom-1*) that is associated with prolonged survival in some mice in our $Apc^{1322T/+}$ mouse colony. We screened mice in this study for the presence of this polymorphic allele. We found 3 mice from the KU-32-treated group and 5 mice from the control group carried the *Mom-1* promoter polymorphisms (see chapter 6), and excluded the data collected from these mice. After excluding these data, we found that KU-32 does not significantly affect liver weight, spleen weight, or blood counts in $Apc^{1322T/+}$ mice. There was an increase in polyp burden (polyp multiplicity and size) in all intestinal regions in KU-32-treated mice compared to the control group. However, this

trend was not statistically significant (figure 7.7 A-D). Interestingly, we found that KU-32 significantly changed the distribution of polyps in $Apc^{1322T/+}$ mice, the same trend we observed in $Apc^{Min/+}$ mice treated with KU-32 or 17-AAG. Polyps in $Apc^{1322T/+}$ mice are distributed more proximally than those in $Apc^{Min/+}$ mice [7]. In KU-32-treated mice, most of the polyps still developed in the jejunum, but the ratio of ileal polyps was almost doubled from 10% to 19%. We conclude that KU-32 affects intestinal tumor distribution in $Apc^{1322T/+}$ mice.

KU-32 protects against colitis associated colon cancer in mice

We have shown that KU-32 alters intestinal tumorigenesis in two different mouse models containing germline mutations in *Apc*. We next tested KU-32 on the AOM-DSS mouse model. In the AOM-DSS model, colon cancer is induced via somatic rather than germline mutations [10]. Unlike $Apc^{Min/+}$ and $Apc^{1322T/+}$ mice which develop tumors mainly in the small intestine, AOM-DSS-treated mice develop colonic tumors. Another advantage of the AOM-DSS mouse model [11] is that *Apc* is usually not mutated, which allows testing the effect of KU-32-induction of full-length Apc on tumor formation. We treated two wild-type C57Bl/6 mouse groups, ten mice each, with a single intraperitoneal injection of 7.5mg/kg AOM at the age of 6 weeks. This was followed by three cycles of 2.5% DSS in drinking water for 5 days at age 7, 11, and 15 weeks. One mouse group received a weekly intra-peritoneal injection of 7.5mg/kg KU-32 starting at ~5.5 weeks (3 days before the AOM injection). The other group received injections of the vehicles only and served as a control (figure 7.8 A). In addition, two other groups, 10 mice per group, received the same KU-32 or vehicle treatment without AOM-DSS, and served as control groups. Body weight was recorded weekly with every injection of either KU-32 or the

vehicle. Two AOM-DSS treated mice on the vehicle had rectal prolapse and were euthanized at the age of 22 weeks. The remaining mice were euthanized at the age of 24 weeks. All mice not treated with AOM-DSS developed no intestinal tumors. These mice gained weight normally, and did not show detectable abnormalities. On the other hand, KU-32-injected AOM-DSS-treated mice gained more weight relative to AOM-DSS treated mice injected with the vehicle only (figure 7.8 B). In addition, KU-32-injected AOM-DSS-treated mice had significantly less colonic tumor incidence and multiplicity (figure 7.8 C-D). While 90% of AOM-DSS-treated mice injected with the vehicle developed at least one colonic tumor, only 60% of the KU-32-treated mice developed colonic tumors. The average number of colonic tumors in KU-32-treated mice was half that in the vehicle-treated mice. In addition, the vehicle-injected mice had significantly more large tumors (≥ 2mm in diameter) relative to the KU-32-treated group (figure 7.8 E). KU-32-injected AOM-DSS-treated mice had significantly fewer lymphoid follicles in their colons compared to the vehicle-treated mice (figure 7.8 F). There was no significant change in the hematological profiles in AOM-DSS-treated groups. KU-32-injected AOM-DSS-treated mice had smaller spleens relative to the vehicle-injected AOM-DSS-treated mice. This same trend was also observed in KU-32-injected mice that were not treated with AOM-DSS, however, which suggests that this effect is not related to colonic inflammation and tumorigenesis (figure 7.8 G).

Discussion

Although the role of APC in protecting from colorectal cancer is well documented, little is known about how APC cellular levels are regulated. In this report, we show that induction of the heat-shock response through heating, treatment with 17-

AAG, or treatment with the novel small molecule KU-32, increases the cellular level of APC. This increase in cellular APC level is not through proteasomal pathway as treating cells with both the proteasome inhibitor MG132 and 17-AAG results in greater increase than with either compound. We think that MG132 might augment 17-AAG increase in APC level by direct and indirect mechanisms. Inhibiting destruction of APC by proteasomes directly increases the cellular APC level. On the other hand, induction of the heat shock response by inhibiting proteasomes could boost the effect of 17-AAG on inducing the heat-shock response.

Although the mechanism by which the heat-shock response upregulates APC is not known, one possible model is that induction of the heat-shock response stabilizes APC protein through upregulation of chaperones. APC interacts with HSP70 through the co-chaperone human homologue of *Drosophila melanoganster* tumorous imaginal disc (hTid) [18; 25]. In addition, Wang *et al*. has shown that APC co-immunoprecipitates with two forms of HSP70 (HSP70-5 and HSP70-2) [18].These data may suggest that HSP70 stabilizes APC. We will further explore this possibility in the future.

As mutations of APC are the initiating step in the majority of colorectal cancer, we expected that increasing the Apc level in mouse intestinal epithelial cells would reduce intestinal tumorigenesis. We used a novel non-toxic compound, KU-32, to increase APC levels in cultured cells and in mouse intestinal epithelial cells. We predicted that upregulation of Apc by use of KU-32 would protect against intestinal tumorigenesis in mice. This prediction was based on the observation that reduction of Apc levels below a certain threshold results in intestinal polyp formation in mice [26; 27], and that upregulation of full-length Apc in mouse intestinal epithelial cells decreased

intestinal polyposis in $Apc^{Min/+}$ mice [28]. We found that KU-32 surprisingly increased the polyp burden in $Apc^{Min/+}$ mice, when the drug was administered at a high dose. One possible explanation of the increased polyp burden in $Apc^{Min/+}$ mice is through upregulation of the truncated Apc^{Min} protein, which has been proposed to have a dominant-negative effect in intestinal carcinogenesis [29]. Supporting this model, we observed an increase in truncated Apc in HT29 cells when cells were heated. We plan to directly test this possibility by treating $Apc^{Min/+}$ mice with KU-32, and monitor truncated Apc level in intestinal epithelial cells in these mice. It is also important to note that although other groups have proposed a dominant negative role of truncated APC in cultured cells [29; 30], there is no evidence for this role in Apc mouse models [7; 31; 32]. Another KU-32 treatment regimen did not change tumor burden in $Apc^{Min/+}$ mice (figure 7.5). These data may suggest that the increase in polyp load by KU-32 is dose-dependent. However, the two KU-32 regimens used in $Apc^{Min/+}$ mice also differ in the mice age at the start and end points and the duration of the treatment. We will investigate possible contributions of these factors on polyp formation in KU-32-treated $Apc^{Min/+}$ mice in the future.

We also treated $Apc^{Min/+}$ mice with an established drug that induces the heat-shock response, 17-AAG. 17-AAG is an HSP90 inhibitor that is in clinical trials as an anti-cancer drug. We reasoned that by comparing the effects of both drugs, we could dissect the effects that are related to the induction of the heat-shock response from those that are drug-specific. We found that 17-AAG decreased polyp size in the stomach and duodenum and colon, an effect that was not observed in $Apc^{Min/+}$ mice treated with KU-32, which suggests that polyp size reduction in 17-AAG-treated $Apc^{Min/+}$ mice is related

to specific effects of 17-AAG, rather than a general effect of induced heat-shock response. We found that KU-32-treated $Apc^{1322T/+}$ mice developed more polyps than did vehicle-treated mice, although not significantly so. These results are inconsistent with our model that KU-32 affects intestinal polyposis in a manner dependent on the particular size of the truncated Apc. However, we have not determined yet if the truncated Apc protein is upregulated in KU-32-treated $Apc^{1322T/+}$ mice.

One of the most intriguing results from this study is that induction of the heat-shock response in17-AAG- or KU-32-treated mice changes polyp distribution. In 17-AAG- and KU-32-treated mice, intestinal polyps were shifted to the ileal region. The regional distribution of polyps in different Apc mouse models is not fully understood (see chapter 2), but it is potentially related to the mechanism of the second *Apc* hit [33]. Induction of heat-shock response might alter the molecular machinery that affects the mechanism of the second *Apc* mutation. An alternative hypothesis is that induction of heat-shock response has difference effects on different intestinal regions, imposing positive or negative selection over the growth of intestinal tumors. Further studies could differentiate between these possibilities.

We have shown that KU-32 protects against colitis-associated colon cancer in the AOM-DSS mouse model. AOM-DSS-treated mice that received weekly injection of KU-32 developed fewer and smaller tumors relative to mice injected with the drug vehicle only. We propose two mechanisms that are not mutually exclusive for this protection against colitis-associated tumorigenesis. KU-32 decreases colitis-associated tumorigenicity through upregulation of Apc. In the AOM-DSS model, Apc is typically not mutated, and tumorigenicity is initiated via stabilizing mutations in β-catenin. [34;

35]. These mutations prevent phosphorylation and degradation of β-catenin by the cytoplasmic destruction complex. However, we propose that upregulation of Apc by KU-32 might decrease β-catenin transcriptional activities by sequestering β-catenin in the nucleus or importing the transcriptional repressor CtBP to the nucleus (see chapter 3). Alternatively, a KU-32-induced heat-shock response might protect the tissue from inflammation. KU-32-treated mice gained more weight and had fewer visible lymphoid follicles in their colons, which are both indicators of a decreased inflammatory process. Future work could differentiate between these two mechanisms.

In conclusion, we show that induction of the heat-shock response increases Apc levels in both cultured cells and intestinal epithelial cells in mice. Induction of the heat-shock response by the novel non-toxic compound KU-32 affects polyp number, size, and distribution in mouse models with germline mutations of *Apc*. KU-32 protects against inflammation-induced colon tumorigenesis in mice with full-length APC.

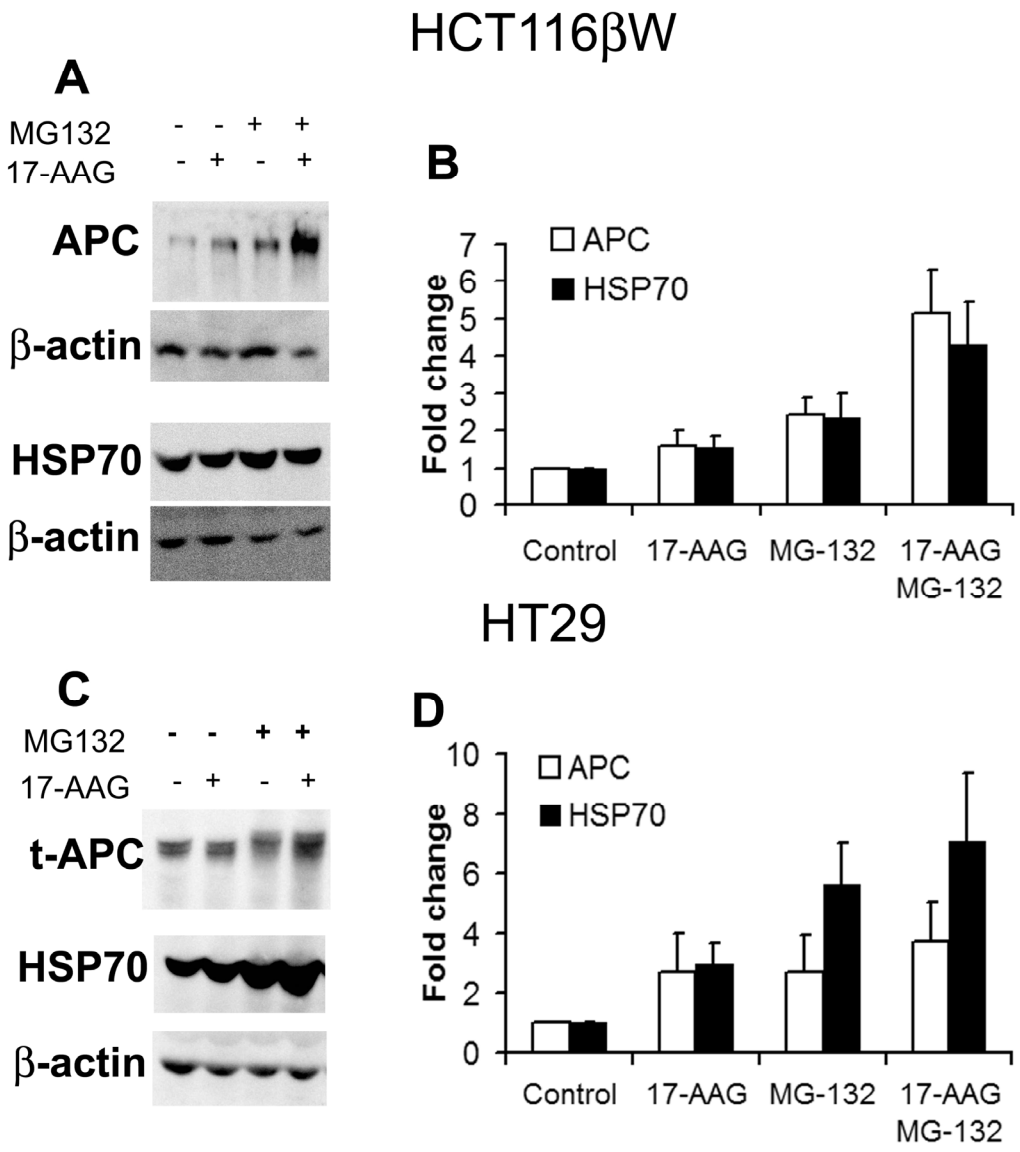

Figure 7.1: 17-AAG and MG132 increases APC and HSP70 levels in colon cancer cell lines. (A) Colon cancer cell lines HCT116 βW with full length APC and **(C)** HT29 with truncated APC (t-APC) were treated with the HSP90 inhibitor, 17-AAG, the proteasome inhibitor, MG132 or with both for 24 hours. Total protein lysates from treated cells were resolved via SDS-PAGE, and Western blots were probed for APC, HSP70, and β-actin, which served as a loading control. **(B & D)** Data from at least five (HCT116 βW cells) and three (HT29 cells) independent experiments are presented as average fold-change of normalized APC or HSP70 band intensity (APC or HSP70/β-actin) compared to untreated cells. Error bars represent SEM.

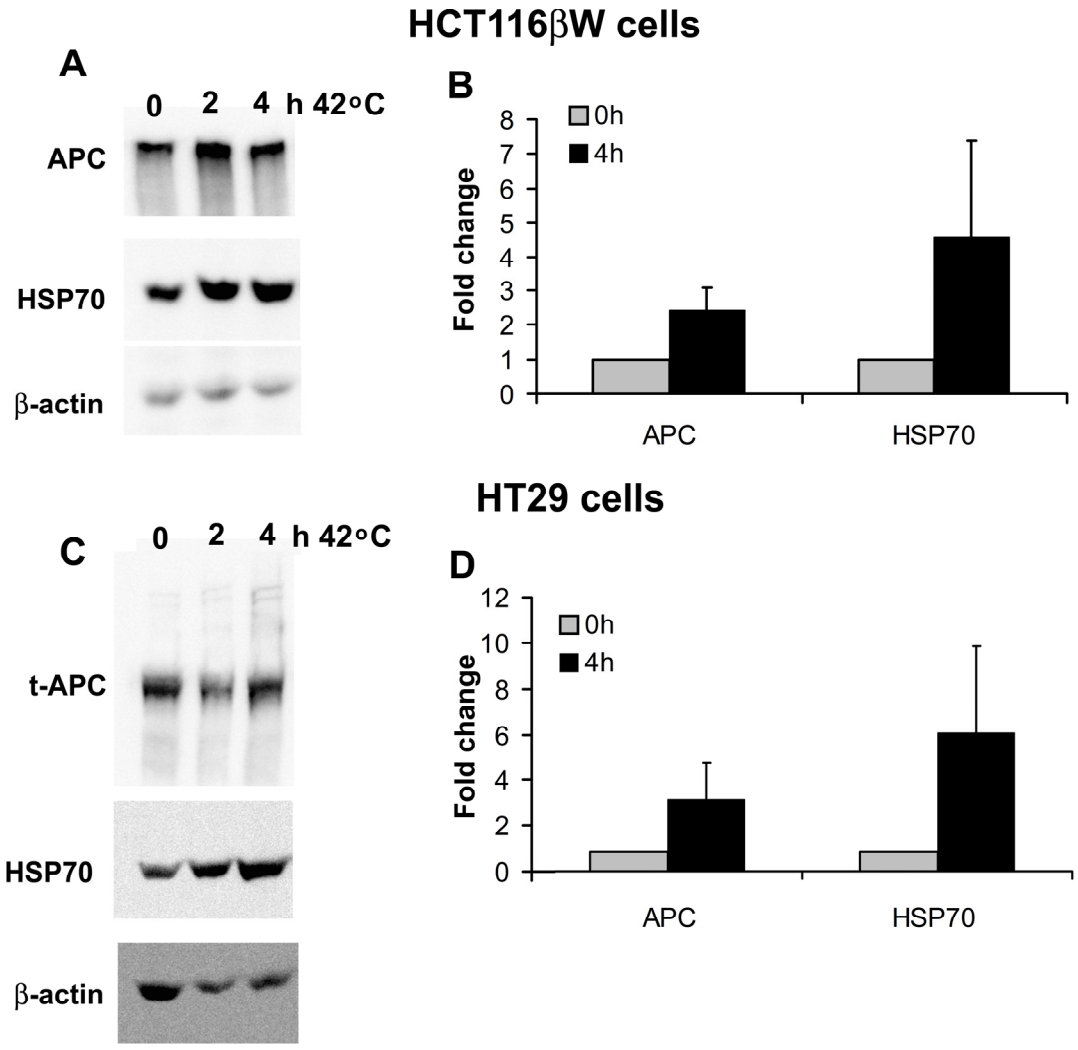

Figure 7.2: **Heat shock increases APC level in colon cancer cell lines (A)** Colon cancer cell lines HCT116 βW with full-length APC and **(C)** HT29 with truncated APC (t-APC) were incubated at 42°C for different times as indicated. Total protein lysates from heated cells were resolved via SDS-PAGE, and Western blots were probed for APC, HSP70, and β-actin that served as a loading control. **(B)** Data from four independent experiments and **(D)** data from 3 independent experiments are presented as average fold change of normalized APC band intensity (APC/β-actin) after heating for 4 hours, compared to cells incubated at 37°C. Error bars represent SEM.

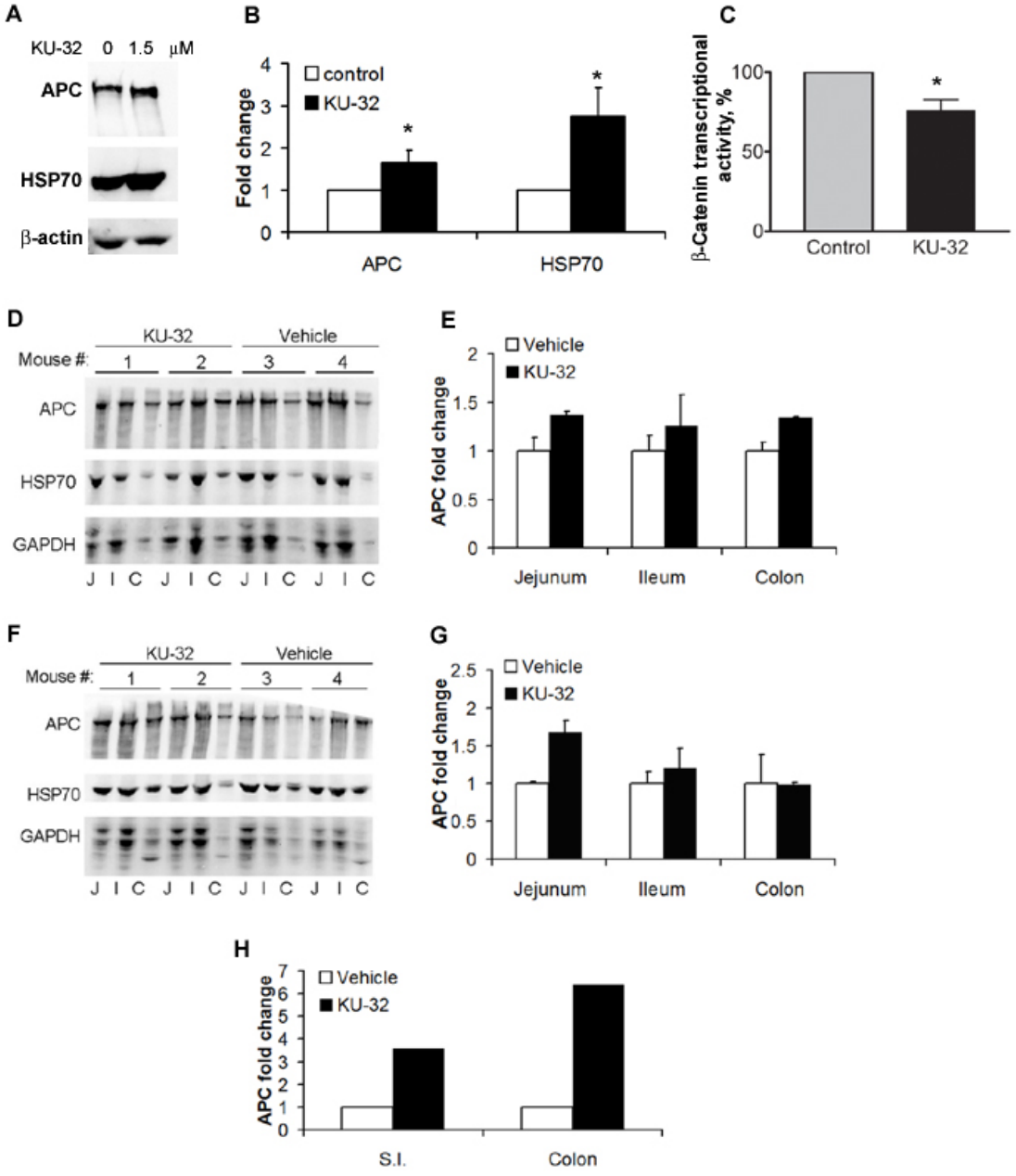

Figure 7.3: KU-32 increases APC level in colon cancer cell lines and *in vivo* in mouse intestinal epithelial cells. (A) HCT116βW cells expressing full-length APC were treated with 1.5 μM KU-32 for 24 hours. Proteins from whole-cell lysates were resolved via SDS-PAGE, and immunoblots were probed for APC, Hsp70, and β-actin, which served as a loading control. **(B)** Data from five independent experiments are presented as average fold change of normalized APC or HSP70 band intensity (APC or HSP70/β-actin) compared to untreated cells. **(C)** HCT116 cells were transiently transfected with the β-catenin reporter TOPFlash or the control reporter FOPFlash. Cells were treated with KU-32 for an additional 24 hours, and the β-catenin activity was determined as

described in materials and methods. Data from three independent experiments are presented as average β-catenin transcriptional activity (TOPFlash-luciferase divided by FOPFlash) of KU-32-treated cells compared to level of untreated cells, which was set to 100% (p=0.04, Mann-Whitney nonparametric test). **(D &F)** Four C57Bl/J mice were injected with either 30mg/kg KU-32 or the drug vehicle alone. After 24 hours (D) and 48 hours (F), epithelial cells were isolated from jejunum (J), ileum (I), and colon (C). Proteins from whole cell lysates were resolved via SDS-PAGE, and immunoblots probed for APC, HSP70, and GAPDH, which served as a loading control. **(E &G)** Data are presented as average fold change of normalized APC band intensity (APC/ GAPDH) from KU-32-injected mice compared to mice injected with vehicle alone. **(H)** A graph showing the fold change in Apc and Hsp70 levels in total protein lysates from mouse intestinal epithelial cells from the small intestine (S.I) and colon after 24 hours of treating the mouse with 7.5 mg/kg KU-32. * indicates $p<0,05$ (Mann-Whitney nonparametric test). Error bars represent SEM.

Mouse model	Drug	Dose	Start age	End age
$Apc^{Min/+}$	KU-32	30 mg/Kg every 2 weeks	2 weeks	14 weeks

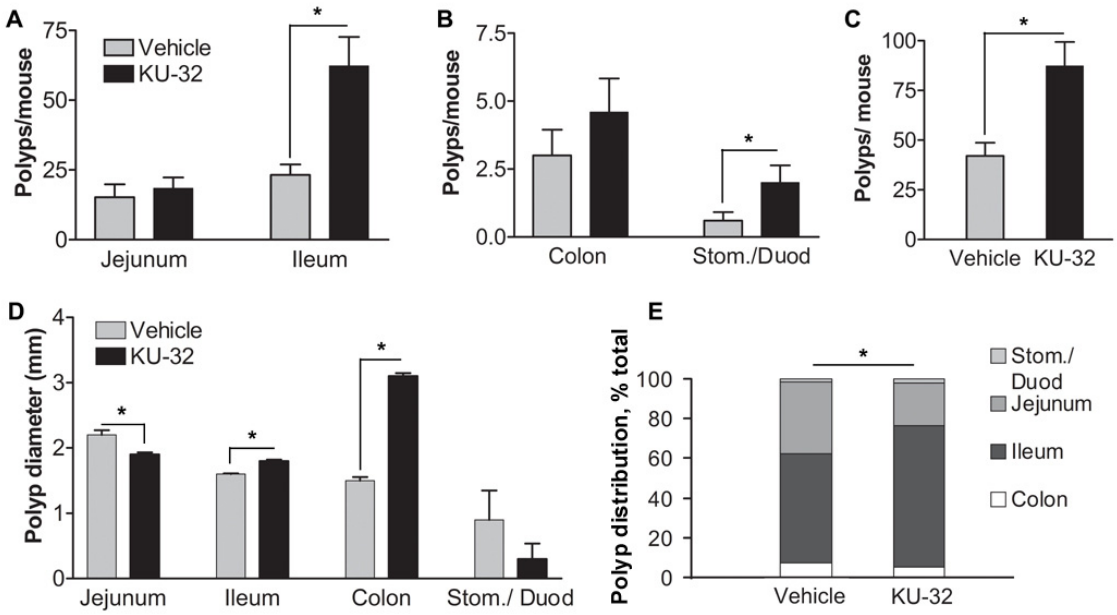

Figure 7.4: A high dose of KU-32 increases polyp burden in $Apc^{Min/+}$ mice. Nine $Apc^{Min/+}$ mice were given seven intra-peritoneal injections with 30mg/ Kg KU-32 every 2 weeks starting at the age of 2 weeks. Ten other $Apc^{Min/+}$ mice were injected with the drug vehicle following the same protocol and served as a control group. (A & B) Average number of polyps in different intestinal regions (C) Average total number of polyps per mouse in both groups of $Apc^{Min/+}$ mice. (D) Average diameter in mm of polyps in different intestinal regions. (E) The distribution of intestinal polyps in different intestinal regions. * indicates $p < 0.05$ Student t-test (A, B, C &D) and Chi-square test (E). Error bars are SEM.

Mouse model	Drug	Dose	Start age	End age
Apc^{Min}	KU-32	7.5 mg/Kg every week	6 weeks	10 weeks

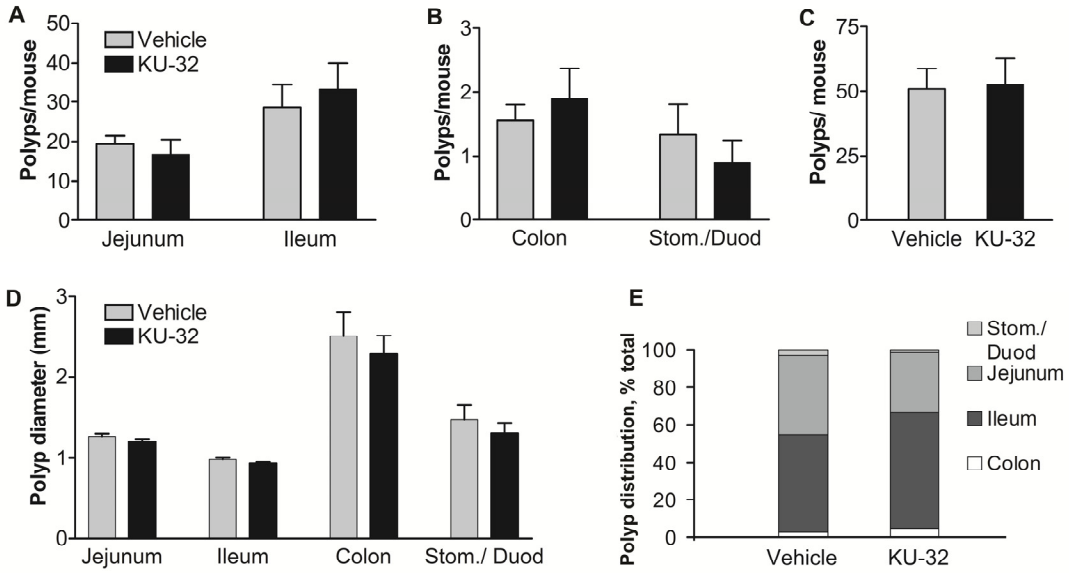

Figure 7.5: A low dose of KU-32 does not increase polyp burden in $Apc^{Min/+}$ mice. Ten $Apc^{Min/+}$ mice were given seven intra-peritoneal injections (7.5 mg/ Kg) of KU-32 every week starting at the age of 6 weeks for 4 weeks. Nine other $Apc^{Min/+}$ mice were injected with the vehicle following the same protocol and served as a control group. (A & B) Average number of polyps in different intestinal regions (C) Average total number of polyps per mouse in both groups of $Apc^{Min/+}$ mice. (D) Average diameter of polyps (in mm) in different intestinal regions. (E) The distribution of intestinal polyps in different intestinal regions. Error bars are SEM

Mouse model	Drug	Dose	Sart age	End age
Apc^{Min}	17AAG	50 mg/Kg 3 times/week	6 weeks	10 weeks

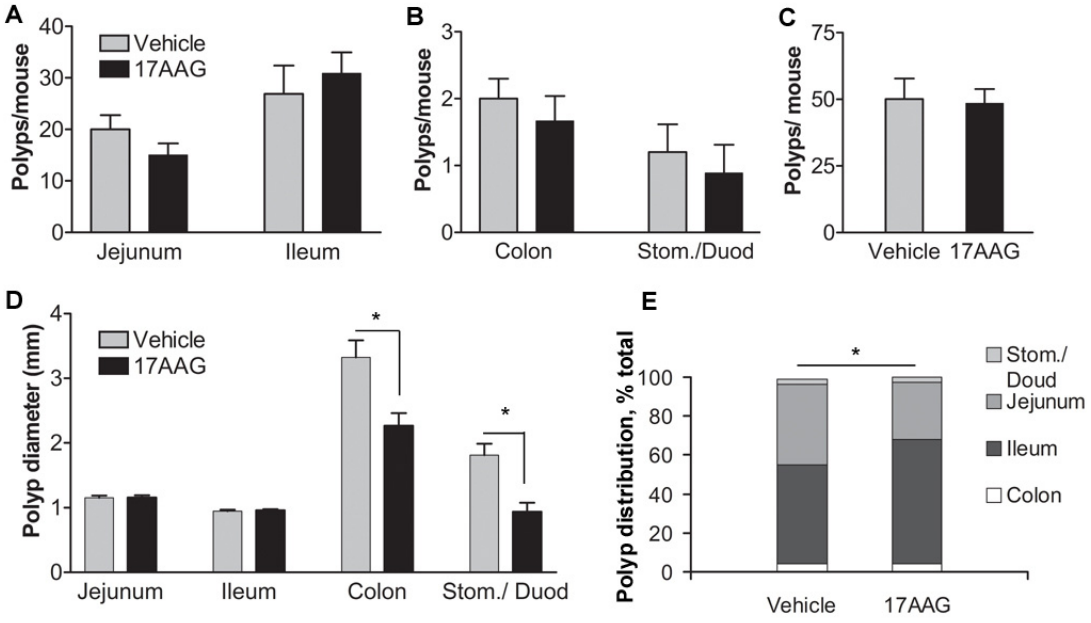

Figure 7.6: 17-AAG affects intestinal polyposis in $Apc^{Min/+}$ mice. Nine $Apc^{Min/+}$ mice were given intra-peritoneal injections of 50mg/ Kg 17-AAG three times a week starting at the age of 6 weeks until the age of 10 weeks. Ten other $Apc^{Min/.+}$ mice were injected with the drug vehicle following the same protocol and served as a control group. (A & B) Average number of polyps in different intestinal regions (C) Average total number of polyps per mouse in both groups of $Apc^{Min/+}$ mice. (D) Average diameter in mm of polyps in different intestinal regions. (E) The distribution of intestinal polyps in different intestinal regions. * indicates $p < 0.05$ Student t-test (A, B, C &D) and Chi square test (E). Error bars are SEM

Mouse model	Drug	Dose	Sart age	End age
Apc^{1322T}	KU-32	7.5 mg/Kg every week	4.5 weeks	9.5 weeks

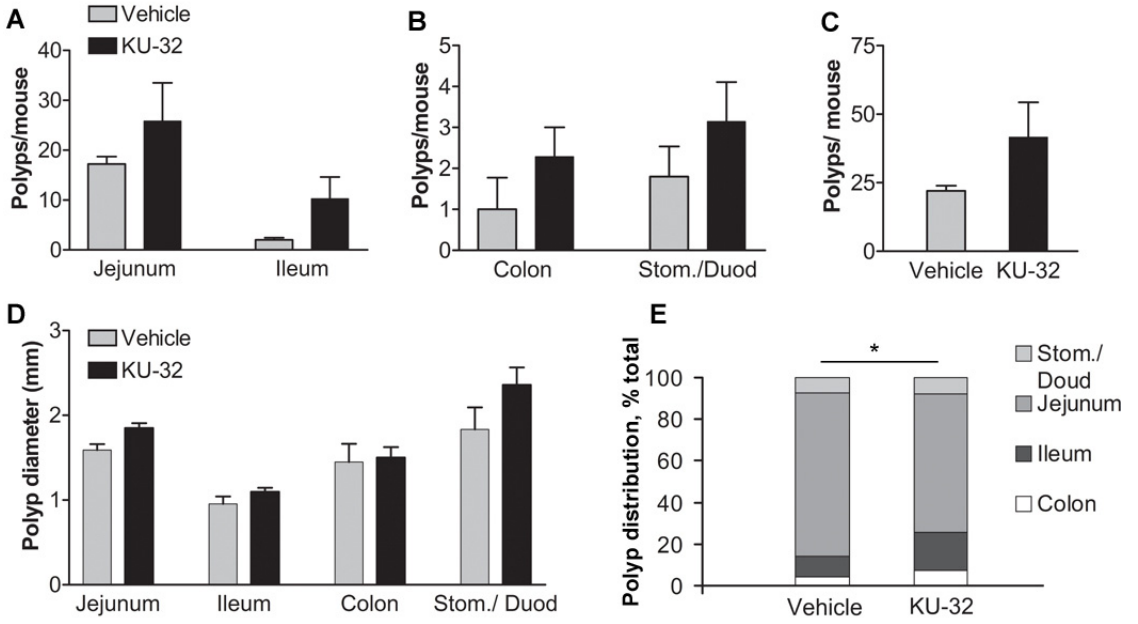

Figure 7.7: KU-32 alters intestinal tumerogenesis in Apc^{1322T}. Seven $Apc^{1322T/+}$ mice were given 5 intra-peritoneal injections of 7.5 mg/ Kg KU-32 every week starting at the age of 4.5 weeks. Five other $Apc^{1322T/+}$ mice were injected with the drug vehicle following the same protocol, and served as a control group. (A & B) Average number of polyps in different intestinal regions (C) The average total number of polyps per mouse in both groups of $Apc^{Min/+}$ mice. (D) Average diameter in mm of polyps in different intestinal regions. (E) The distribution of intestinal polyps in different intestinal regions. * indicates $p < 0.05$ Student t-test (A, B, C &D) and Chi square test (E). Error bars are SEM.

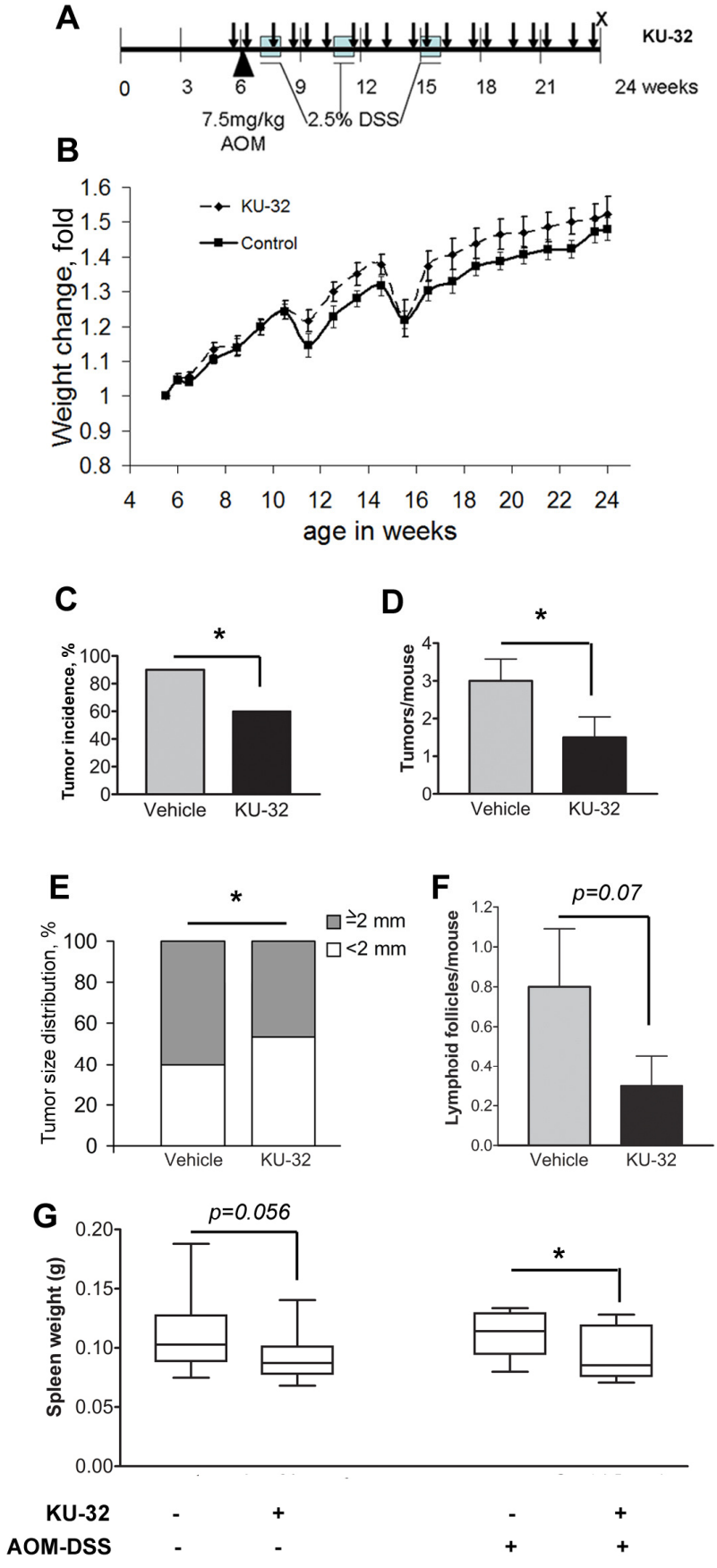
A
KU-32
0
3
6
9
12
15
18
21
24 weeks
7.5mg/kg AOM
2.5% DSS
B
KU-32
Control
Weight change, fold
age in weeks
C
Tumor incidence, %
Vehicle
KU-32
D
Tumors/mouse
Vehicle
KU-32
E
≥2 mm
<2 mm
Tumor size distribution, %
Vehicle
KU-32
F
p=0.07
Lymphoid follicles/mouse
Vehicle
KU-32
G
p=0.056
Spleen weight (g)
KU-32
AOM-DSS

Figure 7.8: KU-32 protects against colitis-associated colon tumorigenesis in mice. **(A)** Ten wild-type mice were administered a weekly dose of 7.5 mg/kg KU-32 (arrows), starting at 5.5 weeks. The mice also received a single dose of AOM (arrowhead) at 6 weeks followed by 3 cycles of 5 days of 2.5% DSS (blocks) in drinking water at age 7, 11 and 15 weeks. The mice were sacrificed at age 24 weeks. Ten other mice received the AOM-DSS treatment, but received a weekly injection of the drug vehicle only, and served as a control group. Two other control groups (10 mice each) received either KU-32 or the vehicle, but not the AOM-DSS treatment. **(B)** KU-32-treated mice gained more weight relative to mice injected with the drug vehicle only. **(C)** Colonic tumor incidence in AOM-DSS treated mice. **(D)** The average number of colonic tumors per mouse in AOM-DSS treated mouse groups. **(E)** The size distribution of colonic tumors in AOM-DSS-treated mice. Polyps are classified into small tumors with diameter less than 2 mm and larger tumors with diameter equal to or exceeding 2 mm. **(F)** The average number of lymphoid follicles per mouse detected under 10X magnification in AOM-DSS-treated mice. **(G)** Spleen weights in different groups included in this study. * indicates $p < 0.05$ Student t-test (D, F, &G) and Chi square test (C &E). Error bars are SEM.

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Chapter 8

Conclusions and future directions

The gatekeeper APC plays a key role in preventing colorectal carcinogenesis [1]. APC is best known for its role in antagonizing Wnt-signal-mediated cellular proliferation, by forming a cytoplasmic complex that targets the oncoprotein β-catenin for proteasomal degradation [2]. APC shuttles between the cytoplasm and the nucleus, aided by two nuclear localization signals and five nuclear export signals. Several reports showed a role for nuclear APC in Wnt signaling, transcription regulation, DNA replication, and DNA repair. However, these studies were performed using cultured cells and purified proteins [3]. In this dissertation work, we characterized the phenotype of a mouse model that was made in our lab. In this mouse model, the ability of APC to go to the nucleus is hindered by mutations in both nuclear localization signals (Apc^{mNLS}). We propose that the Apc^{mNLS} mouse model will expand our understanding of nuclear functions of Apc within the whole organism. This model will also allow us to test the physiological relevance of nuclear APC functions identified from use of other models such as cultured cells and purified proteins. For instance, we found that $Apc^{mNLS/mNLS}$ mice have high expression of Wnt target genes, and increased proliferation in the intestinal epithelial cells, which supportsthe hypothesis that nuclear Apc antagonizes Wnt signaling.

Based on studies performed in cultured cells, nuclear APC was proposed to antagonize Wnt signaling via three possible mechanisms: 1- nuclear APC binds to and exports nuclear β-catenin to the cytoplasm [4, 5];2- Nuclear APC sequesters β-catenin in the nucleus [6], and; 3- APC facilitates nuclear import of the transcriptional repressor CtBP, and thus decreases β-catenin transcriptional activity [7]. We did not find changes

in β-catenin localization in cells from $Apc^{mNLS/mNLS}$ mice, which is inconsistent with the first mechanism. Future work using $Apc^{mNLS/mNLS}$ mice will differentiate between the latter two mechanisms. For example, determining the amount of β-catenin bound to promoters of Wnt target genes, or interacting with TCF/LEF, as determined by means of chromatin immunoprecipitation (ChIP), might allow us to validate the second proposed mechanism. Similarly, measuring changes in nuclear localization of the transcriptional suppressor CtBP, by means of immunostaining or cellular fractionation, will allow validation of the third mechanism.

CRC-associated *APC* mutations result in loss of the C-terminal part of APC, including both nuclear localization signals [8]. As these mutations also disrupt other APC domains, including β-catenin binding and degradation domains, it is difficult to determine the contribution of NLS_{APC} to tumorigenesis. Using $Apc^{mNLS/mNLS}$ mice, we showed direct evidence that nuclear Apc suppresses tumorigenesis. Apc^{mNLS} increases intestinal and mammary tumorigenesis in $Apc^{mNLS/Min}$ mice relative to the number of tumors in $Apc^{Min/+}$ mice. We do not completely understand the mechanism of enhanced tumorigenicity afforded by the Apc^{mNLS} allele. However, increased Wnt signaling and proliferation, as we demonstrated in intestinal tissue, may play a role in the mammary tissue as well. Moreover, we showed that intestinal polyps in $Apc^{mNLS/Min}$ mice show loss of the Apc^{mNLS} allele. As discussed in detail in chapter 5, these results are consistent with the Apc^{mNLS} allele increasing the rate of LOH in intestinal epithelial cells. Using fluorescence *in situ* hybridization (FISH) and array-comparative genomic hybridization (CGH), we will determine if the Apc^{mNLS} allele increases chromosomal instability in cells from $Apc^{mNLS/Min}$ mice. Alternatively, nuclear Apc may have a role in inducing tumor

growth in a cell-non-autonomous manner. To test this proposed mechanism, we could isolate living epithelial cells from intestinal polyps from $Apc^{Min/+}$ mice as described [9], or use embryonic stem cells homozygous for the *Apc*^{Min} allele [10]. We will implant these cells in the walls of the large intestine of $Apc^{Min/+}$ and $Apc^{mNLS/Min}$ mice, and determine if the genotype of the surrounding tissues affects the growth rate of these cells. As $Apc^{Min/+}$ and $Apc^{mNLS/Min}$ mice are congenic C57Bl/6J animals, we do not expect immunological rejection of the implanted cells.

In addition, $Apc^{mNLS/mNLS}$ mice are more sensitive to colitis-induced tumorigenesis in the AOM-DSS model. We also showed that $Apc^{mNLS/mNLS}$ mice have higher expression of the inflammatory mediators *Cox2* and *MIP2* in intestinal epithelial cells from $Apc^{mNLS/mNLS}$ colons, which may contribute to the enhanced tumorigenesis in these mice. We plan to explore these possibilities and directly test the role of nuclear Apc in inflammation. We will induce acute inflammation in the colons of $Apc^{mNLS/mNLS}$ and $Apc^{+/+}$ mice using a higher dose of DSS, and will assess the degree of colonic inflammation both pathologically and biochemically.

Although much work has been done to understand APC, little is known about how cellular APC levels are regulated. In the second part of this dissertation, we showed that cellular APC levels are increased with induction of the heat-shock response. In the future, we will investigate the mechanism underlying this APC protein increase. We will differentiate between two not necessarily mutually exclusive, mechanisms: heat shock response increases APC level via upregulation of *APC* transcription; or the increase occurs through stabilization of APC protein. We will measure *APC* transcription in

response to induction of the heat-shock response by measuring mRNA levels, or using a reporter assay.

We found that KU-32, a novel non-toxic compound that induces the heat-shock response, increases APC levels both in cultured cells and in mouse intestinal epithelial cells. We explored the effect of KU-32 on intestinal tumorigenicity in two mouse models with different germline mutations in Apc ($Apc^{Min/+}$ and $Apc^{1322T/+}$ mice). We also tested another small molecule that induces the heat-shock response, 17-AAG, in $Apc^{Min/+}$ mice. We did not detect prophylactic properties of these drugs on intestinal tumorigenesis in mice with germline mutations in *Apc*. However, we found that KU-32 and 17-AAG change intestinal polyp distribution in $Apc^{Min/+}$ and $Apc^{1322T/+}$ mice. As discussed in detail in chapter 2, changes in intestinal polyp distribution in Apc mouse models might represent differential changes in different aspects of intestinal tumorigenicity, including the mechanism of loss of the *Apc* wild-type allele, cellular proliferation, and apoptosis. Future work will elucidate the contribution of these mechanisms in altering intestinal polyp distribution in Apc mouse models treated with KU-32 and 17-AAG, by assessing LOH, proliferation indices, and apoptosis in polyps from different intestinal regions.

We found that KU-32 reduces colitis-associated colon tumorigenesis in the AOM-DSS mouse model. It is not clear if this KU-32-mediated protective effect requires upregulation of wild-type Apc, or is mediated by other aspects of the induced heat-shock response. Further studies will delineate the contribution of Apc to this protection by assessing the protective effects of KU-32 in inflammation-induced tumorigenesis in Apc mutant ($Apc^{Min/+}$) mice. We will also test the contribution of HSP70 in protection from

colitis-mediated tumorigenesis, by testing KU-32 in AOM-DSS-treated HSP70 knockout mice [11].

In conclusion, findings described in this dissertation contribute to understanding several aspects of APC biology. First, they demonstrate a role for nuclear Apc in antagonizing Wnt signaling, suppressing intestinal proliferation, and suppressing intestinal and extra-intestinal tumorigenesis. Second, these findings support a novel mechanism by which cellular Apc levels are regulated: induction of the heat-shock response. Finally, we show that induction of the heat-shock response alters intestinal tumorigenesis in mice. We think that this line of work will ultimately contribute to improving preventive, diagnostic, prognostic, and therapeutic measures for colorectal cancer.

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